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CLINICAL STUDY OF CEREBRAL SMALL VESSEL DISEASE

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RESUMEN

La enfermedad de pequeño vaso cerebral (EPVC) o microangiopatía cerebral (MAC) son dos términos equivalentes para denominar la afectación de la microcirculación cerebral. La definición de microcirculación cerebral o pequeño vaso incluye a arteriolas, vénulas y capilares con calibres de entre 5 micro milímetros y 2 milímetros de diámetro.

Hay diferentes tipos de EPVC, las más frecuente son las formas esporádicas, conocidas como angiopatía amiloide y la arteriopatía hipertensiva, también llamada arterioloesclerosis o arteriopatía relacionada con la edad o con los factores de riesgo vasculares.

Otras formas esporádicas están relacionadas con procesos inflamatorios o autoinmunes sobre la microcirculación cerebral, como la granulomatosis de Wegener, el síndrome de Churg-Strauss, la poliangeítis microscópica y otras vasculitis asociadas a enfermedades del tejido conectivo.

Existen formas genéticas menos frecuentes, por ejemplo la arteriopatía autosómica dominante con infartos subcorticales y leucoencefalopatía (CADASIL), su forma autosómica recesiva conocida como CARASIL, la enfermedad de Fabry, la EPVC causada por mutaciones del COL4A1, la vasculopatía hereditaria cerebro-retiniana (CRV), la retinopatía hereditaria vascular (HVR) y otras conocidas también por su acrónimo en ingles como el HERNS o el MELAS.

La angiopatía amiloide es producida por el depósito de β amiloide (βA), una proteína insoluble, en los vasos sanguíneos, en forma de fibrillas. Se observa en pacientes con Enfermedad de Alzheimer (EA) y en pacientes ancianos. Hay formas genéticas vinculadas a mutaciones en el gen de la proteína precursora del amiloide, en el gen de la presenilina y en el síndrome de Down.

La incidencia de la angiopatía amiloide aumenta con la edad. El depósito de βA se observa hasta un 40% de los cerebros de

pacientes ancianos e incluso se estima que es mayor en aquellos mayores de 90 años.
La arteriopatía hipertensiva o arteriosclerosis suele ser una enfermedad sistémica que afecta también a la microcirculación de otros órganos como la retina y el riñón.
La etiología es atribuida a la disfunción endotelial producida por el envejecimiento exacerbado por la acción de los factores de riesgo vasculares, principalmente la hipertensión arterial y la diabetes mellitus.

Revisaremos brevemente las características anatomopatológicas y la patogénesis de las dos principales formas de EPVC.

1. Angiopatía amiloide: se produce afectación de las arterias leptomeníngeas por el depósito de βA que ocurre inicialmente alrededor de las células musculares lisas en la capa media y adventicia de las arteriolas; progresivamente se va produciendo la destrucción de esta capa y se observan fenómenos degenerativos como la degeneración hialina con engrosamiento de la pared vascular y reducción de la luz vascular (llegando incluso a la oclusión del vaso sanguíneo), formación de microaneurismas y necrosis fibrinoide. La necrosis fibrinoide se produce por la trasudación de plasma hacia la pared arteriolar por la ruptura de las células endoteliales afectadas y se caracteriza por la acumulación de un material granular eosinofílico consistente en depósitos de fibrina, inmunoglobulinas e inmunocomplejos.

El péptido de βA es el producto del proceso proteolítico de la proteína precursora de amiloide que es una glicoproteína transmembrana localizada en las neuronas. De este proceso surgen diferentes isoformas que se diferencian en función del número de aminoácidos que lo constituya. Las isoformas más importantes son la 1-40, 1-42 y 1-38. Los péptidos de 40 aminoácidos de βA son más solubles que los de 42 aminoácidos, por lo que el βA 1-40 tiende a acumularse en los vasos sanguíneos. El βA 1-42 sin embargo, juega un papel fundamental en la patogénesis de la EA, ya que se agrega en forma de micro-fibrillas que se acumulan en las placas neuríticas.

El βA 1-40 producido es drenado a través de los espacios perivasculares. Los espacios perivasculares contienen liquido

intersticial y constituyen vías de eliminación de productos de deshecho del metabolismo celular hacia liquido cefalorraquídeo y posteriormente hacia el sistema linfático. Esta distribución de drenaje a través de los espacios perivasculares hacia el espacio subaracnoideo de las arterias leptomeníngeas explica la localización topográfica de la afectación por la angiopatía amiloide donde se observan fenómenos lobares y corticales.

La angiopatía amiloide puede considerarse una angiopatía por un fallo de eliminación, en este caso del péptido βA 1-40; el fallo resulta en la acumulación y la formación de agregados en los espacios perivasculares.

La acumulación en estos espacios de βA 1-40 produce además activación de la inflamación, disfunción mitocondrial, aumento del estrés oxidativo con aumento de la fosforilación de tau (otro mecanismo fuertemente implicado en la patogénesis de la EA) y ruptura de la barrera hemato-encefálica (BHE). Estos mecanismos conducen a la neurotoxicidad celular, desencadenando apoptosis.

2. Arteriopatía hipertensiva: las alteraciones histopatológicas observadas son cambios degenerativos observados en los fenómenos de aterosclerosis. La aterosclerosis afecta a las arterias perforantes que irrigan ganglios basales, tálamo y tronco cerebral. Es una enfermedad inflamatoria que conduce al engrosamiento y endurecimiento arterial que se inicia mediante el proceso de disfunción endotelial. Se observan fenómenos de lipohialinosis, necrosis fibrinoide, formación de microateromas y microaneurismas. La lipohialinosis se caracteriza por el depósito de colágeno y de mucopolisacáridos en forma de una masa amorfa que engruesa la lámina basal de las arteriolas. Los microaneurismas se forman por la pérdida de pericitos y la ruptura de la BHE y de la pared microvascular.

El daño microvascular se produce por la presencia de factores de riesgo vasculares, principalmente por la hipertensión y la diabetes que producen disfunción endotelial y degeneración de la pared vascular.

No podemos hablar de microcirculación cerebral sin hacer referencia a la unidad neurovascular y a la barrera hemato-encefálica. Los componentes de la unión neurovascular son

neuronas, astrocitos, las células endoteliales y su membrana basal.
La unión de la capa de células endoteliales y su membrana basal constituye lo que conocemos como BHE.

Las células endoteliales de los capilares se encuentran unidas entre si mediante unas uniones moleculares muy especiales que restringen la permeabilidad capilar; llamadas uniones estrechas. A su vez mantienen uniones con pericitos que mantienen la estabilidad capilar y se anclan en la lámina basal formada principalmente por colágeno. En esta estructura de unión de las células endoteliales a la lámina basal intervienen las metaloproteasas, cuya disfunción implica ruptura de la BHE, alteración de la permeabilidad, inflamación y neurotoxicidad.

A su vez las células nerviosas interactúan con la BHE, que se encuentra unida a astrocitos y neuronas. Los astrocitos realizan funciones de regulación de la homeostasis, señalización celular, estabilización de las uniones estrechas y transmiten la señalización de neuronas e interneuronas que regulan la hemodinámica cerebral en función del metabolismo neuronal (mecanismo conocido como autorregulación astrocito-neurona del flujo sanguíneo cerebral).

La disfunción de la BHE con el aumento de la permeabilidad vascular es uno de los mecanismos fundamentales en la enfermedad de pequeño vaso tal y como establecen los trabajos de J. Wardlaw publicados por primera vez en 2009. Las metaloproteasas con actividad proteolítica sobre las uniones estrechas, son liberadas en respuesta a fenómenos de isquemia y de disfunción endotelial provocados por la arterioesclerosis y por el depósito de βA en la pared vascular.

Existe un mecanismo sinérgico entre ambos tipos de angiopatía ya que la pérdida de la pulsatilidad vascular y de la integridad de la pared por aterosclerosis favorece la acumulación de βA en los espacios perivasculares.

La manifestación clínica de la enfermedad de pequeño vaso se produce años después del desarrollo de estos procesos. A través de técnicas de neuroimagen, principalmente de resonancia magnética cerebral nos ha permitido conocer diferentes formas o fenotipos de manifestación de la enfermedad.

Los principales fenotipos son la leucoaraiosis, los infartos lacunares, las microhemorragias y las hemorragias intraparenquimatosas.

La leucoaraiosis o lesión de sustancia blanca se define como la disminución de atenuación o hipodensidad en TC cerebral o bien la hiperintesidad en secuencias de T2 o FLAIR en RM cerebral localizada en la sustancia blanca periventricular; estas estructuras son regiones frontera de la circulación cerebral y profunda. En relación a la naturaleza de estas lesiones, se considera que son producidas por desmielinización, pérdida axonal y por fenómenos de isquemia.

Los infartos lacunares son cavidades o pequeños agujeros < 15 mm, producidos por isquemia de arterias perforantes de la circulación profunda cerebral principalmente por fenómenos de lipohialinosis, otras causas menos frecuentes son los embolismos de origen cardíaco o ateroembólico.

Las microhemorragias son pequeños depósitos de hemosiderina formado por productos de degradación de la sangre localizados en macrófagos y que causan un artefacto de susceptibilidad detectado en secuencias específicas (SWI y T2*). Los microaneurismas son el origen de estas MH y también de las hemorragias intracraneales. Las MH que están localizadas en los ganglios basales o en el tronco cerebral están relacionadas con la microangiopatía hipertensiva y las de localización subcortical con la angiopatía amiloide.

Desde el punto de vista clínico la enfermedad de pequeño vaso se manifiesta con deterioro cognitivo de perfil frontosubcortical, con menor afectación de la memoria pero con disfunción ejecutiva, inatención, labilidad emocional, y alteraciones visu-espaciales; alteraciones en la marcha, del equilibrio e incontinencia urinaria y con el desarrollo de depresión. La angiopatía amiloide se vincula más con un perfil de deterioro cognitivo de perfil amnésico típico de la Enfermedad de Alzheimer.

En la década de los años 2000 se desarrollan estudios epidemiológicos para estudiar la influencia de los factores de riesgo vasculares en la enfermedad de pequeño vaso, principalmente la hipertensión arterial, pero también la diabetes mellitus tipo 2 y de otros no modificables como la edad.

Destacamos los trabajos de Schmidt en el Austrian Stroke Prevention Study, el de Vermeer et al en el Rotterdam Scan Study, el de Dufoil et al en el The PROGRESS Study, el de Gouw et al en el LADIS y los trabajos de Coordonier en el ámbito de las microhemorragias.

Tras estos estudios se establece una asociación importante entre la hipertensión arterial y la edad con la progresión y desarrollo de la enfermedad de pequeño vaso cerebral. En relación a la influencia de la diabetes, la dislipemia y de otros factores de riesgo vasculares los estudios son menos concluyentes.

En la última década se han producido importantes avances en el conocimiento de la fisiopatología de esta entidad. Especial relevancia tiene los siguientes procesos: 1) la disfunción de la unidad neurovascular; 2) la ruptura de la barrera hemato-encefálica a través de la acción de metaloproteasas y 3) el depósito de B amiloide 1-40 en los vasos sanguíneos por procesos de disminución de su aclaramiento a través de los espacios perivasculares por disfunción endotelial y disminución de la pulsatilidad arteriolar producidos por aterosclerosis.

Como resumen de la **justificación de este trabajo** destacamos que en este estudio se pretende estudiar la enfermedad de pequeño vaso como un único desorden fisiopatológico (aunque también se analizan los diferentes fenotipos por separado), en fases precoces o subclínicas de la enfermedad e investigar aspectos fisiopatológicos vinculados a mayor progresión en el tiempo; para poder determinar marcadores precoces de la enfermedad y anticiparse previniendo el daño vascular y neurodegenerativo y así evitar las serias secuelas que produce.

El principal objetivo es identificar un perfil clínico de riesgo asociado con la progresión de la enfermedad de pequeño vaso, los **objetivos secundarios** consisten en identificar biomarcadores relacionados con deterioro cognitivo (AB 1-40), disfunción endotelial (sTWEAK) y disfunción de la matriz extracelular y ruptura de la BHE (Metaloproteasas).

En relación a los **aspectos metodológicos** se ha diseñado un estudio prospectivo seleccionando pacientes sin enfermedad de pequeño vaso conocida pero con alto riesgo de desarrollarla. Para

ello, se han incluido pacientes de más de 60 años de edad con historia previa de más de 5 años de evolución de hipertensión arterial y/o diabetes mellitus tipo 2.

Se han definido estos criterios en base a las guías actuales de hipertensión y diabetes. Se excluyeron pacientes con las siguientes características: 1) historia previa de AIT, ictus isquémico o hemorragia intracraneal; 2) historia de enfermedad vascular previa; 3) diagnóstico previo de deterioro cognitivo o demencia; 4) fuente cardíaca embolígena, 5) cáncer o enfermedad sistémica grave con expectativa de vida menor a 5 años; 6) enfermedad inflamatoria crónica; 7) inclusión en otro estudio; 8) negativa del paciente a participar en el estudio.

Los criterios para la interrupción del seguimiento son los siguientes: 1) abandono o retirada del consentimiento informado; 2) aparición de eventos vasculares (cardiacos, neurológicos, oftalmológicos o sistémicos).

El tiempo de seguimiento fue mayor de un año en cada caso.

Se analizaron variables clínicas, neurorradiológicas, cognitivas y ultrasonográficas asociadas con la progresión de la enfermedad de pequeño vaso. La variable principal del estudio es la aparición o la progresión de cualquiera de los fenotipos de enfermedad de pequeño vaso en el tiempo: leukoaraiosis, infartos lacunares, microhemorragias y deterioro cognitivo. Las variables secundarias del estudio son la aparición o incremento de cada uno de los fenotipos durante el seguimiento.

Los pacientes fueron seleccionados en centros de atención primaria de Porto do Son (A Coruña) y A Estrada (Pontevedra) y posteriormente derivados al Hospital Clínico Universitario de Santiago de Compostela. Los pacientes fueron revisados cada 6 meses en su centro de salud y anualmente en el Hospital.

En los centros de atención primaria se evaluaron y recogieron datos relativos a la edad, género, datos antropométricos y demográficos como la talla, el peso, el índice de masa corporal (IMC); datos de la historia médica y tratamientos habituales recibidos analizando los siguientes 1) historia previa de hipertensión arterial y su grado de control en base a criterios clínicos y de monitorización ambulatoria de la presión arterial (MAPA) de 24 horas; 2) historia previa de DM2 y su grado de control; 3) historia previa de dislipemia y su grado de control. Se

recogieron muestras de sangre para determinación de hemograma, bioquímica y coagulación y específicamente los siguientes parámetros: HbA1c, perfil lipídico, función renal y albuminuria, VSG y fibrinógeno.

Se realizó un ECG de 12 derivaciones para descartar la presencia de una fibrilación auricular.

En el ámbito hospitalario se recogieron datos relativos a la evaluación cognitiva utilizando el Minimental State Examination (MMSE), el Addenbroke´s Cognitive Examination (ACE) en su versión adaptada a una comunidad rural gallega (utilizando la versión de Caballero et al) y la escala de depresión geriátrica de Yessavage. Se analizó con estos datos el perfil de deterioro cognitivo obtenido (sugestivo de EA, de perfil vascular o mixto). La progresión de deterioro cognitivo se definió como la disminución de puntuación en los test (de forma mantenida) en realización de los test sucesivos.

La evaluación neurorradiológica se llevó a cabo utilizando resonancia magnética cerebral de 1,5 Tesla. Se analizó la presencia y progresión de la leukoaraiosis utilizando la escala de Fazekas (I o leve, II o moderada y III o severa); la presencia y desarrollo de nuevos infartos lacunares; y la presencia y desarrollo de nuevas microhemorragias.

Se realizó también una evaluación ultrasonográfica de troncos supra aórticos para estudio de placas de ateroma y estudio de estenosis hemodinámica; y de arterias intracraneales midiendo el índice de pulsatilidad y resistencia en la arteria cerebral media. A través de métodos de ELISA se llevaron a cabo determinaciones basales, a los 12 meses y a los 24 meses de sTWEAK, βA 1-40, TIMP-1, y de las metaloproteasas MMP-1, MMP-10, MMP-7, MMP-9, MMP-12, MMP-13 y MMP-3.

Se calculó el tamaño de la muestra en base a estudios previos en los que se estima que el 30% de la población hipertensa de más de 60 años de más de 5 años de evolución desarrolla progresión de leucoaraiosis en 3 años de seguimiento. Por lo que se calcula una población entre 90 y 130 sujetos. Los análisis estadísticos fueron realizados con el programa IBM®SPSS® statistics v.20 para Mac.

Se utilizó un análisis multivariado de regresión logística con el objetivo de analizar variables que estuviesen independientemente asociadas con la progresión de la enfermedad de pequeño vaso, progresión de deterioro cognitivo, progresión de leukoaraiosis, con el desarrollo de nuevos infartos lacunares y de nuevas microhemorragias. Las odds ratio (OR) se ajustaron por las variables que fueron significativas en el análisis bivariado. Los resultados fueron expresados como OR ajustadas con los correspondientes intervalos de confianza (95% IC). Los valores de p por debajo de 0,05 fueron considerados estadísticamente significativos.

Los **resultados** más relevantes fueron los siguientes:

1) En relación a las **características basales de la muestra**:

Se evaluaron 207 pacientes entre 2013 y 2016; 106 fueron excluidos por presentar algún criterio de exclusión.

Se incluyeron en el estudio 101 pacientes y durante el seguimiento se perdieron 9 pacientes. El tiempo medio de seguimiento fue de 24±4,86 meses. La edad media fue de 71±5 años. El 59,4% fueron mujeres. El 92,1% tenían sobrepeso u obesidad. El 99% de los pacientes eran hipertensos en el momento de la inclusión; el 57,4% tenia mal control clínico de la tensión arterial y el 20,8% mal control en el MAPA de 24 horas. El 36,6% de la muestra tenia diabetes tipo II en el momento de la inclusión; el 45,7% con mal control glucémico. El 74,3% de la muestra presentaba dislipemia en el momento de la inclusión; un 34,6% con mal control.

Respecto a la neuroimagen basal, un 87,1% presentaba algún grado de leukoaraiosis, infartos lacunares un 9,9%, microhemorragias un 10,9% y deterioro cognitivo un 21,8%. Los pacientes presentaban algún marcador basal de enfermedad de pequeño vaso en un 87,1% de los casos.

En relación a las variables ultrasonográficas se detectó un patrón patológico (sugestivo de microangiopatía) del IP en el 73,3% y del IR en el 57,4%.

Durante el seguimiento se observó progresión de cualquier fenotipo en un 42,6% de los pacientes. Un 12,9% presentaron

progresión de deterioro cognitivo, un 28,7% progresión de leukoaraiosis, un 14,9% nuevos infartos lacunares y un 14,9% nuevas microhemorragias

2) Variables asociadas de forma independiente a la **progresión de cualquier fenotipo de la enfermedad de pequeño vaso:**

Las variables que se asociaron a progresión de cualquier fenotipo durante el seguimiento fueron el IMC como factor protector (OR 0,84; 95% CI 0,73-0,87) y el mal control de la HTA (OR 4,73; 95% CI 1,18-18,91). En relación a las variables de laboratorio; de forma discreta se observa que los pacientes con niveles más elevados de βA 1-40 a los 24 meses de seguimiento desarrollaron con mayor probabilidad progresión de cualquier fenotipo de la enfermedad (OR 1,05; 95% CI 1,00-1,10).

3) Variables asociadas de forma independiente a la **progresión de deterioro cognitivo:**

No se objetivaron variables clínicas asociadas a la progresión de deterioro cognitivo. En relación a las variables de laboratorio; los pacientes con niveles más elevados de βA 1-40 a los 24 meses desarrollaron deterioro cognitivo con mayor probabilidad que el grupo de pacientes con niveles más bajos (OR 1,02 CI 95% 1,00-1,03).

4) Variables asociadas de forma independiente a la **progresión de leucoaraiosis:**

La variable clínica que se asoció a progresión de leucoaraiosis durante el seguimiento fue el mal control de la HTA (OR 7,8; 95% CI 1,5-40,4). No se encontraron variables de laboratorio asociadas a progresión de leukoaraiosis.

5) Variables asociadas de forma independiente al **desarrollo de nuevos infartos lacunares:**

La variable clínica que se asoció al desarrollo de nuevos infartos lacunares fue la presencia de nuevas microhemorragias (OR 20,1; 95% CI 3,0-135,1). La variable de laboratorio asociada al desarrollo de nuevos infartos lacunares fueron niveles elevados de sTWEAK a los 24 meses (OR 1,11; 95% CI 1,06-5,17).

6) Variables asociadas de forma independiente al **desarrollo de nuevas microhemorragias**:

Ninguna variable clínica se asoció con el desarrollo de nuevas microhemorragias, sin embargo las variables de laboratorio que se asociaron fueron los niveles elevados de MMP-7 a los 24 meses (OR 1,85 95% CI 1,03-2,32) y los niveles elevados de MMP-9 a los 24 meses (OR 1,21 95% CI 1,00-3,12).

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ABBREVIATIONS

AA: Arachidonic acid
ABCB1: ATP-binding cassette sub-family B member 1
ABMP: Ambulatory BP monitoring
ABI: Ankle-brachial index
ACR: Albumin-creatinine ratio
ACE: Angiotensin
ACE: Addenbrooke`s Cognitive Examination
ACT: Antichymotrypsin
AMI: Acute myocardial infarction
ANOVA: Analysis of variance
ApoE: Apolipoprotein E
ApoJ: Apolipoprotein J
AQP4: Aquaporin-4
α2M: Alpha 2 macroglobulin.
Aβ Amyloid βeta
APP: Amyloid precursor protein
ACE-1: Angiotensin converting enzyme-1
ACh: Acetylcholine
AD: Alzheimer disease
AQP4: Aquaporin-4
AT: Angiotensin.
ATP: Adenosine triphosphate
AJ: Adherens junction
TJ: Tight junction
BACE: β-site amyloid precursor protein cleaving enzyme
BBB: Blood-brain barrier
BH4: Tetrahydrobiopterin
BK Ca: Calcium activated potassium channels
BM: Basal membrane
BMI: Body mass index.
BP: Blood pressure
CAA: Cerebral amyloid angiopathy

CADASIL: Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CARASIL: Cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy
CaV: Voltage-operated calcium channels
CBF: Cerebral blood flow
CCD: Couple-charged device
CD: Cluster of differentiation
cGMP: Cycling guanine monophosphate
CMA: Cerebral microangiopathy
CMB: Cerebral microbleeds
CMI: Cerebral microinfarcts
CNS: Central nervous system
COX-2: Cicloxigenase-2
CPP: Cerebral perfusion pressure
CSF: Cerebrospinal fluid
CSVD: Cerebral small vessel disease
CRP: C reactive protein
CRV: Cerebroretinal vasculopathy
CT: Computer tomography
DLP: Dyslipidemia
DM2: Diabetes mellitus type 2
EDHF: Endothelium-derived hyperpolarizing factor
ECs: Endothelial cells
ECG: Electrocardiography
ELISA: Enzyme-Linked ImmunoSorbent Assay
EPCs: Endothelial progenitor cells.
eNOS: Endothelial NO synthase
FTD: Frontotemporal dementia
EM: Extracellular matrix
EPVS: Enlarged perivascular spaces
FGF: Fibroblast grow factor
GLUT-1: Glucose transporter protein type 1
HERNS: Hereditary endotheliopathy with retinopathy nephropathy and stroke.
HIFα: Hypoxia inducible factor alpha

HT: Hypertension
HRP: Horseradish Peroxidase
HVR: Hereditary vascular retinopathy
ICAM-1: Intercellular adhesion molecule 1.
ICP: Intracranial pressure
ICH: Intracerebral haemorrhages
IL1β: Interleukin 1 β
IMT: Intima media thickness
iNOS: Inducible nitric oxide synthase
ISF: Interstitial space fluid
Kcs: Potassium channels
LA: Leukoaraiosis
LACI: Lacunar infarct by OCSP classification
LAM: Leukocyte adhesion molecule
LEG: Light Emitting Diodes
LI: Lacunar infarcts
LRP: Low density lipoprotein receptor-related protein
PEFA: Protein elimination failure angiopathy
MCP-1: Monocyte chemoattractant protein
MDR-1: Multidrug resistance protein1
MMPs: Matrix metalloproteinases
MMSE: Mini-Mental State Examination
MRP: Multidrug resistance proteins
MH: Microhemorrhages
MRI: Magnetic resonance imaging
MTHFR: Methylenetetrahydrofolate reductase
NADPH: Nicotinamide adenine dinucleotide phosphate
NF κ B: Nuclear factor kappa-light chain enhancer of activated B cells
NO: Nitric oxide
nNOS: Neural nitric oxide synthase
NVU: Neurovascular unit
OCSP: Oxfordshire Community Stroke Project
OD: Optical density
OGTT: Oral glucose tolerance test
OR: Odds ratio

PEFA: Protein elimination failure angiopathy
PDGF: Platelet derived growth factor
PDGF β: Platelet derived growth factor β
PCs: Pericytes
PG: Prostaglandin
PET: Positron emission tomography
PI: Pulsatility index
PICALM: Phosphatidylinositol binding clathrin assembly protein
PON: Paraoxonase
PrP: Prion protein
PSEN: Presenilin
PVSs: Perivascular spaces
RAGE: Receptor for advanced glycation end-products
RI: Resistant index
ROS: Reactive oxidative specie
SGC: Soluble guanylyl cyclase
SBI: Silent brain infarcts
sTWEAK: Soluble TNF-related weak inducer of apoptosis
SVD: small vessel disease
SWI: Susceptibility weighting imaging
TACE: Tumor necrosis alpha converting enzyme
TEER: Trans-endothelial electrical resistance
TGFβ: Transforming growth factor beta
TIA: Transient ischemic attack
TIMPs: Tissue inhibitors of matrix metalloproteinases
TMD: Transmembrane domain
TNF α: Tumor necrosis factor alpha
TJ: Tight junctions
TOAST: Trial of ORG in Acute Stroke Treatment
TRAF: TNF receptor associated factor
TTR: Transthyretin
PECAM-1: Platelet endothelial cell adhesion molecule
PVS: Perivascular space
VaD: Vascular dementia
VCAM-1: Vascular cell adhesion molecule 1
VCI: Vascular cognitive impairment

VLA-4: Very late antigen 4 or Integrin α4β1.
VE: Vascular endothelium
VEGF: Vascular endothelial Growth Factor
VLOM: Verbal fluency + language / orientation + memory as a deferred recollection
VRS: Virchow Robin space
WML: White matter lesions
WMH: White matter hyperintensities
ZO: Zonula occludens.
5-HT: 5-hidroxitriptamina

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DE SANTIAGO
DE COMPOSTELA

INTRODUCTION

1. BACKGROUND AND OVERVIEW

Cerebral small vessel disease (CSVD), small vessel disease (SVD) or cerebral microangiopathy (CMA) are equivalent terms to refer to the affectation or damage of the small arteries, arterioles, venules, and capillaries of the brain. It comprises a set of diseases with diverse etiologies. The most prevalent forms are arterioloesclerosis related to hypertension and ageing, and cerebral amyloid angiopathy (CAA) (1).

In this pathology, radiological phenotypes acquire high transcendence for its diagnosis. Furthermore, radiological aspects are the main way to detect CSVD in subclinical phases. Four different clinical and radiological phenotypes are recognized in CSVD (although the phenotypes usually coincide in the same patient): leukoaraiosis (LA), microhemorrhages (MH), most of the lacunar infarcts (LI) and a considerable proportion of spontaneous intracerebral hemorrhages (ICH) (2).

CSVD has a crucial role in stroke, principally in lacunar stroke and ICH, but beyond its role in clinically overt acute stroke syndromes, CSVD causes widespread microvascular damage which has a cumulative effect on cognition, impaired mobility and gait disturbance (3). CSVD is currently the main cause of vascular dementia (VaD) (4) and it has an important role in the ageing. CSVD also causes psychiatric problems such as depression in elderly patients. Studies in this regard have found a higher incidence of depressive symptoms in patients with magnetic resonance imaging (MRI) markers of CSVD and high association with white matter lesions (WML) severity, aging and physical disabilities corresponding to geriatric syndromes like gait disturbances, falls, incontinence, sensory impairment and functional impairment (5)(6).

The intense relationship between CSVD and age will involve an increase in the prevalence of this disease in the coming decades, with the increase of life expectancies.

CSVD was recognized in the 19th century. The first mentions were made by Binswanger and Alois Alzheimer, recognized German neuropathologists, who described multiple infarcts and chronic

ischemia in autopsy studies of brains affected by ¨senile dementia¨, concept associated to age since the Greco-Roman period (7).

Binswanger described these findings in 1894 initially focused on differentiating clinical and pathological aspects of syphilitic general paralysis that was very frequent at that time, from progressive cognitive and physical decline with memory loss, apathy, disinhibition and focal findings such as aphasia, hemiparesis or hemianopsia, apoplectic attacks and periods of stabilization that happened during the course of the illness. Binswanger named this condition *encephalitis subcorticalis chronica progressive* and it was characterized by " a relative well preserved of the cortex but with high damage of white matter with atrophy, unusually narrow, gray, and studded with patches, ventricles were enlarged and small arteries and venules also showed signs of fatty degeneration" (8).

Binswanger disease was a term initially used by Alzheimer and colleagues to describe the cases of subcortical arteriosclerotic encephalopathy and it is maintained today to refers to subcortical vascular dementia (9).

Until the mid-twentieth century senile dementia was thought to be primarily caused by cerebral atherosclerosis. But later studies demonstrated that Alzheimer's disease (AD) lesions were much more frequent than chronic ischemia. This vascular etiology was challenged by the studies of Blessed, Tomlinson, and Roth (10), which established that AD, rather than vascular pathology as the main cause of dementia in late life and thought that when vascular pathology was responsible for dementia it was done through the occurrence of multiple infarcts (11).

Jerzy Olszewski, Canadian neuropathologist, published in 1965 two cases of subcortical arteriosclerotic encephalopathy, expanding and defining the pathological and clinical features of this clinical entity (12). Moreover he studied the properties of blood-brain barrier (BBB) such as permeability of cerebral blood vessels using different substances (13).

At the same time, the next major advance in the pathophysiology of CSVD was carried out by Miller Fisher in the 1960s, with special attention to vascular damage around WML. Fisher described the

classical lacunar syndromes. He was also interested in underlying vascular pathology in lacunar lesions. In 1979 he studied ten patients with LI and observed the lumens of penetrating arteries of internal capsule, and in nine cases, he found obstructive vascular lesions such as atheroma or embolism and did not find vascular occlusions in the two rest cases (14,15).

Other works, mainly by French researchers were carried out around the concept of lacunes several years ago. In fact, the term lacunae is introduced by Dechambre in 1838 as "small cavities during the process of resorption within cerebral sotftenings", later Durand-Fardel used this term and in 1843 described the "état criblè" as "several small holes in the white matter and basal ganglia of brain hemispheres associated to dementia or delirium".

Later, in 1901, it was established the distinction between lacunae caused by occlusion of the blood vessels by an arteriosclerotic process (known as softening way) and the one caused by a perivascular space; dilatation that causes destruction of the adjacent brain parenchyma, (known as destructive vaginalitis way) (16).

Studies related to Cerebral Amyloid Angiopathy (CAA) were developed from the beginning of the 20th century following a similar evolution. Amyloid-βeta (Aβ) protein deposition in the cerebral blood vessels was first observed by Gustav Oppenheim in 1909 who studied autopsies of brains with senile dementia and pathological alterations of AD (17). Scholz published in 1938 a work focusing on the vascular alterations observed in the affected vessels that today we know as Cerebral Amyloid Angiopathy (18).

In 1954, Pantelakis called this type of cerebral angiopathy produced by amyloid deposition, the Congophilic Angiopathy, and described several of its main characteristics such as the deposit in medial and adventitia of small leptomeningeal and cortical arteries and capillaries without adjacent parenchymal involvement, and moreover described the main pathological features of CAA such as the location in occipital lobe specially, the association with advanced age and dementia and lack of association with hypertension and arteriosclerosis (19). Histological diagnosis of CAA requires the use of special staining

for amyloid, the Congo-red stain, first synthesized in 1883 and the standard method of amyloid staining (20).

In 1975 Mandybur showed a high incidence of CAA in brains affected by AD in postmortem studies (21).

The works published in the 70s by Okazaki, which analyzed cases of cerebrovascular amyloidosis in the Mayo Clinic, conclude that there is an important relationship with lobar ICH (22). Multiple cortical infarctions and large and recurrent parenchymal hemorrhages were observed. It was described the typical vascular affectation with wall disruption, thickening and narrowing as well as fibrinoid degeneration and miliary aneurysm formation. It was also described the double-barreled vessel wall appearance caused by cracking of the arterial media. Additionally, parenchymal damage with perivascular and senile plaques was observed and capillary deposits often infiltrate the surrounding parenchymal tissue with degenerative neurites.

The investigation of drainage systems of the brain towards the cerebrospinal fluid (CSF) and lymphatic pathways began in the 1980s (23). Studies of the processes of the elimination of waste proteins, (including Aβ) were carried out and later, the concept of protein elimination failure proteins angiopathy (PEFA) arises, where CAA is included. The role of perivascular spaces such the pathways that drive these proteins towards the lymphatic vessels, that were finally discovered in the brain recently, in 2018 (24,25).

With the development of neuroimaging techniques such as computed tomography (CT) and MRI at the end of the twentieth century the high prevalence of chronic microangiopathy damage was appreciated and the interest in this disease resurfaced (26).

Hachinski, a Canadian neurologist born in Ukraine, with numerous researches in dementia and stroke, described in 1987 the term leukoaraiosis (LA), Greek term that means white matter changes or rarefaction of the white substance (27). It is a descriptive word to explain the white matter attenuation observed in subcortical areas near to ventricles in form of diffuse periventricular rims and caps around horns of the lateral ventricles and focal WML in corona radiate, centrum semiovale, and subcortical white matter. These alterations are commonly found in patients with VaD. Furthermore Hachinski created

a scale (Hachinski scale) to differentiate by clinical features VaD from AD (28).

In point of fact LI, LA, MH and ICHs are easily detected by neuroimaging, that is why normally the term CSVD is frequently used to describe the parenchyma lesions detected by neuroimaging, radiological and clinical phenotypes rather than the underlying small vessel alterations (2).

The association and the influence of vascular risk factors, mainly the hypertension, but also diabetes mellitus and others non modifiable risk factors such aging, in the development of these diseases has been mainly studied through epidemiological studies developed in the early 2000s. We should highlight the works of Schmidt et al in the Austrian Stroke Prevention Study, Vermeer et al in the Rotterdam Scan Study, Dufoil et al in The PROGRESS study, Gouw et al in the Leukoaraiosis and Disability Study and Coordonier et al in the field of microbleeds (29–33).

There have been very interesting advances in the last ten years in the understanding of the physiopathology of this entity. Johanna Wardlaw and colleagues, from Edinburgh, published in 2009 that the underlying cause of CSVD is the rupture and dysfunction of BBB and it does not occur mainly through ischemic mechanisms by thrombi or vascular occlusions of the perforating arteries as had been thought until then. The pathological process that constitutes CSVD is a diffuse process in which vascular lesions such as segmental arteriolar disorganization, lipohialinosis segmentaria and necrosis fibrinoid are caused by endothelial dysfunction and breakdown of the blood-brain barrier. Several substances like proteins, inflammatory biomarkers and red blood cells leave the capillaries towards the perivascular space resulting in vascular damage and perivascular edema. In fact the presence of enlarged perivascular space is an early biomarker of CSVD (34–37).

Proteases and free radicals are released by the inflammatory cells in the process of remodeling of the damaged vessels by the influence of molecules such as hypoxia inducible factor 1 alpha (HIFα) that is released by hypoxia secondary to alterations in vascular circulation (38). These proteases and free radicals disrupt and break down the

fibrotic basal lamina and tight junctions proteins and finally attack myelinated fibers, producing by-stander demyelination (35, 36).

BBB dysfunction has been well documented in many studies of leukoaraiosis, lacunar infarcts, advanced aging, cognitive impairment of vascular cause and also in AD (41).

The deposition of Aβ in the vessels of patients with AD could explain the vascular damage observed in cases of late onset AD, since it causes endothelial dysfunction and rupture of the BBB. In turn vascular damage produces greater Aβ peptide accumulation since adequate arteriolar pulsatility and vascular wall integrity are necessary to favor the efflux of perivascular drainage towards lymphatic system; these studies were carried out by Carare et al in 2013 (42,43).

In the introduction of this work a bibliographic review will be carried out about the characteristics of the cerebral microcirculation, neurovascular unit and BBB and the pathology, radiological and clinical characteristics of the two main forms of CSVD: arteriolosclerosis microangiopathy and amyloid microangiopathy.

2. MICROCIRCULATION: ANATOMY AND FEATURES

2.1 ARTERY SYSTEM

Cerebral circulation is divided in macro-circulation and micro-circulation depending on the size of the vessel size. On the arterial side, macro-circulation includes carotid and vertebral arteries and extends to pial arteries. The microcirculation is formed by pial (or leptomeningeal) and penetrating or perforator arterioles, the capillaries and the venules.

The brain receives around 20% of the total blood; in fact it is the organ that receives more amount of blood in the organism, needing a constant and stable perfusion. (44)

The arterial blood supply to the human brain is based on the right and left internal carotid and the right and left vertebral arteries **[Figure 1].**

The internal carotid arteries vascularize the cerebrum; the two vertebral arteries become the basilar artery more distally and their branches vascularize cerebellum and brain stem.

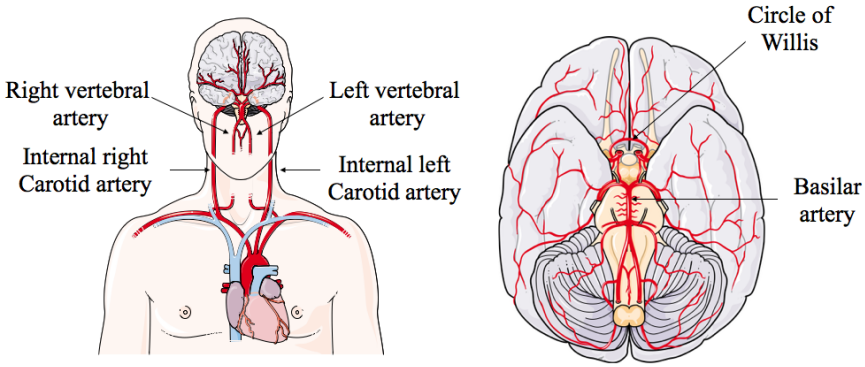


Figure 1: Brain circulation: is formed by the right and left internal carotid and the right and left vertebral arteries and intracranially by basilar artery and circle of Willis and their main branches. Own figure (based on references in the section 2.1)

At the base of the skull the internal carotid arteries and the basilar artery form a special system of collateral arteries called the polygon of Willis, shaped like a ring. Polygon of Willis is formed by six branches the right and left anterior, right and left, and posterior cerebral arteries. Between these main arteries there are communicating arteries, anterior communicating artery and posterior communicating arteries.

These cerebral arteries are progressively divided into smaller arteries and arterioles called “penetrating or perforator arteries” that

vascularize deep areas of the parenchyma. Other branches are situated in more superficial areas to supply blood to the corresponding regions of the cerebral cortex (45) **[Figure 2].**

White matter of the cerebrum and basal ganglia receives its blood-supply through two types of penetrating or perforator arteries: superficial penetrators arterioles that arise from the pial (leptomeningeal) vessels and deep or basal perforators that arise from the large vessels at the base of the brain (46).

Superficial arterioles give two types of penetrating arteries, the cortical arteries that vascularize the cerebral cortex with short branches called cortical arteries and the medullary arteries that vascularize the subcortical white matter.

Deep arterial perforators at the base of the brain vascularize the basal ganglia, thalami and brainstem structures (47).

Penetrating arteries are located in the arachnoid space and are surrounded by CSF, their branches (the arterioles), penetrate into the brain within the perivascular spaces (PVS) or Virchow-Robin spaces (VRS), a continuation of the subarachnoid space (48).

Penetrating arteries give rise to the parenchymal arterioles in their route towards deeper territories of the cerebral parenchyma. The capillaries derived from those arterioles are ensheathed by astrocyte end-feet (49).

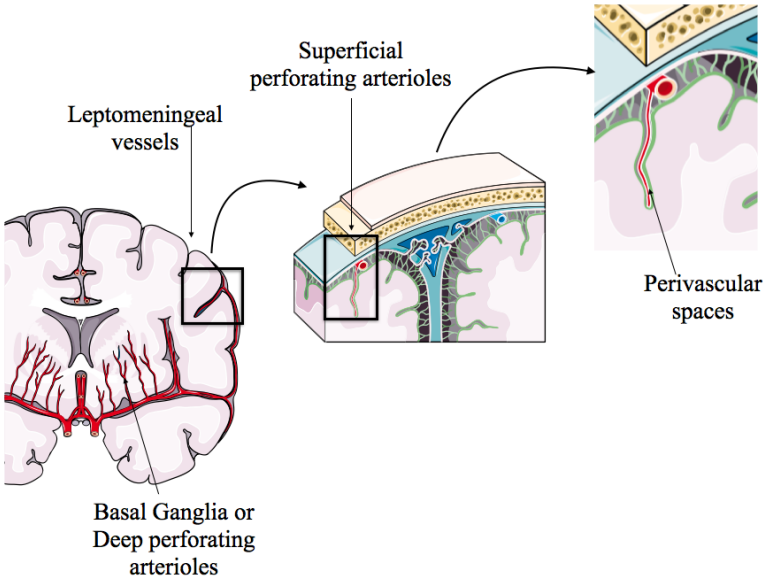


Figure 2: Penetrating arteries and leptomeningeal arteries: for the vascularization of basal ganglia and deep white matter (periventricular) and gray matter of cortical and subcortical white matter. Own figure (based on references in the section 2.1)

The terminals of the astrocytes cover the synapses but also portions of the cerebral circulation; in fact, they covert almost 99% of the vascular surface of capillaries, venules and arterioles. These terminal structures that surround the cerebral microcirculation are denominated astrocyte end-feet or glio-vascular end-feet (50,51). The pial arteries are not covered by astrocytic cells but receive an innervation of the autonomic peripheral nervous system.

2.2. HISTOLOGY:

The wall of cerebral arteries and arterioles has three concentric layers; from internal to external; tunica intima consists of a single layer of endothelial cells and the internal elastic lamina, tunica media consists mainly of smooth muscle cells and also by some elastin and collagen

fibers and the outermost layer, tunica adventitia formed by collagen, fibroblast, perivascular nerves and astrocytic end-feet.

Endothelium in the brain is very specialized and form the BBB. BBB has unique properties of the microvasculature of the central nervous system (CNS) that tightly regulates the exchange of nutrients, solutes and water between the brain and the blood (52).

As the arteries become smaller in relation to the caliber of the vessel and are introduced into the cerebral parenchyma, they are losing thickness of muscle cells, therefore smaller pial arteries have 2-3 layers and penetrating and parenchymal arterioles contain a single layer.

Cerebral arteries have not external elastic lamina unlike systemic arteries (53) which may influence their response to alteration in luminal pressure as compared to arteries elsewhere in the body. Other feature of the cerebral arteries is the lack of vasa vasorum and their nutrition derive from CSF (54).

2.3 VENOUS SYSTEM:

Veins of the brain have no muscular tissue in their walls and they don´t have valves like veins in the rest of the organism. Venous system leaves the brain parenchyma into the subarachnoid space, cross the arachnoid mater and the meningeal layer of the dura mater and drain into the cranial venous sinuses.

The drain system is formed by superficial cortical veins, or pial veins, located and deep or central veins.

Pial veins are located in the pia matter on the surface of the cortex. They drain the cerebral cortex and subcortical white matter and comprise superior sagittal sinuses and cortical veins. This venous system has a unique architecture (55), and unlike the paired arterial-venous arrangement found in many organs in the body, pial veins are not accompanied by pial arteries.

Deep or central veins consists of lateral sinus, straight sinus and sigmoid sinus along with draining deeper cortical veins that they drain into the brain including deep white and gray matter surrounding the lateral and third ventricle or the basal cistern. Venous outflow from the

superior sagittal sinuses and deep veins is directed via confluence of sinuses toward the sigmoid sinuses and Jugular veins (56).

Venules do not have a vascular wall composed clearly by three layers (endothelium, media and adventitia) like arteries and arterioles because they do not have typical smooth muscle cells (55). The venous endothelium is part of BBB and it is a frequent place of BBB breakdown in response to aggression (infection, acute hypertension…) (57).

2.4 COLLATERAL FLOW

Brain circulation contains a system of vascular networks or collateral circulation that is a true redundancy arterial supply for the maintenance of cerebral blood flow in situations of occlusion or vasoconstriction.

The main system is the Circle of Willis that provides low resistance connections and therefore allows reverse flow to provide blood to proximal occluded arteries.

There is also a system of collaterals at leptomeningeal space that provides flow in distal occlusions of cerebral artery of the Circle of Willis. Though penetrating and parenchymal arterioles when are occluded induced an ischemia in the surrounding tissue because they are long and largely unbranched arteries (58).

2.5 PERIVASCULAR INNERVATION

Large and small pial arteries and arterioles have a perivascular innervation that contact with the adventitial layer; it is originated from the peripheral nervous system or also called " extrinsic system" and is formed by superior cervical ganglia, sphenopalatine ganglion, otic ganglion and trigeminal ganglion.

Sympathetic nervous via arises from the superior cervical ganglia with primary neurotransmitters norepinephrine and neuropeptide Y, substances with vasoconstrictive function (59). The sphenopalatine and otic ganglia form the parasympathetic nervous via containing vasoactive intestinal peptide, acetylcholine, nitric oxide synthase and the trigeminal ganglion have sensory afferent nerves containing

calcitonin gene-related peptide substance P, neurokinin A, and pituitary adenylate-cyclase activating polypeptide, substances with vasodilator effect (60).

As the entrance to the cerebral parenchyma takes place and the spaces of VRS are vanishing, these arterioles lose the contribution of extrinsic innervation and receive a contribution of the own central nervous system through neurons located in the brain, this system is called "intrinsic system" for this reason. These neurons are situated in Locus Coeruleus, Raphe Nucleus, Nucleus Basalis and respectively contain norepinephrine, serotonin or 5-hidroxitriptamina (5-HT) and acetylcholine (ACh) and also nitric oxide (61).

There are cortical GABA-interneurons that elicited changes in local microvessel diameter for subcortical basal forebrain ACh and brain stem 5-HT afferents. These interneurons could act as relays to adapt perfusion to local changes in activity following afferent signals from subcortical pathways (62).

Interneurons and subcortical neurons project terminals to astrocytes surrounded the arterioles; this fact is anatomically best described as a neurovascular unit or better defined "neuronal-astrocytic-vascular" tripartite unit (49).

2.6 MICROCIRCULATION AND " NEURO -VASCULAR UNIT"

Capillary network in the brain is composed of specialized endothelial cells and lacks smooth muscle. It is the main place for exchange of oxygen and nutrients, and it is estimated that for each neuron there is a capillary in a 1:1 ratio (63).

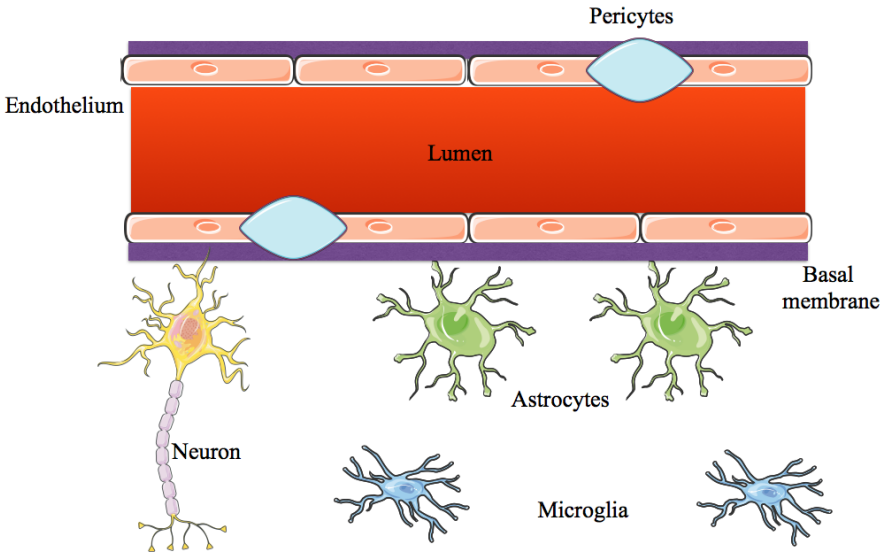


Figure 3: Neurovascular unit. Own figure (based on references in the section 2.6)

The structure of the capillary network is unique compared to other organs. Endothelial cells and pericytes are enclosed by a basal lamina containing type IV collagen, heparin sulfate proteoglycans, laminin, fibronectin and other extracellular matrix proteins; also the basal lamina is surrounded by astrocyte end-feet that ensheathes the capillary.

The components of this structure present an intimate contact, functional interactions and signaling. They establish a complex cross-talking between them; therefore , it is known as neurovascular unit (64,65). The components of the neurovascular unit are neurons, astrocytes interneurons, endothelial cells and extracellular matrix **[Figure 3]**.

The neural control of the microcirculation is carried out through these astrocytes that establish bridges between the neurons and the capillaries.

The structure, anatomically is the astrocyte end-foot, that involves the capillaries. Neurons, via interneurons can modulate the contribution of oxygen and nutrients to certain regions of the brain that need a higher metabolism, by means of a hyperemia that triggers calcium signaling in astrocytes and lead changes in vascular tone (vasoconstriction or vasodilatation).

Other molecules implicated are prostaglandin E and nitric oxide with vasodilator effect and adenosine with vasoconstrictor effect (66), and the signals that mediated the beginning of the process seem to depend on extracellular K concentration and stimulation of glutamate receptors (67). This process is known as functional hyperemia (68).

The mechanism of autoregulation, in other words, the capacity of microcirculation to maintain a constant blood flow is also regulated by this neurovascular coupling (62).

3. BLOOD-BRAIN BARRIER

The first references to a special barrier , that separates the brain from other compartments such as systemic circulation date from the beginning of the 20th century. Ehrlich and Goldmann´s research using different types of colorants injected in the systemic circulation or in the cerebrospinal fluid demonstrated the permanence of theses in only one system (the systemic circulation or the cerebrospinal fluid without being able to transfer to the other compartment) indicating the existence of a non-permeable barrier. The term of blood-brain barrier (BBB) or hematoencephalic barrier was proposed by Lewandowsky in 1900. However, until the 1960s, with the development of electron microscopy, its structure could not be studied (69,70).

Nowadays, it is known that BBB of the brain microvasculature has unique properties that regulates CNS homeostasis. Capillary endothelium of the brain is continuous and does not have pores or

fenestrations and it has other properties like tight junctions (TJ), high proportion of pericytes, and the interaction with astrocytes; these properties produces high selectivity to exchange and movements of molecules, ions and cells from circulation to brain.

The endothelial cell layer and its basement membrane constitute the BBB (71).

3.1 ENDOTHELIAL CELLS

Endothelial cells (ECs) are modified simple squamous epithelial cells and derived from the mesoderm. In the brain the ECs form a single layer in the finer capillaries and are thinner than in other organs such as the muscle (72). ECs are together through molecular junctions (tight junction and adherens junction) forming a polarized endothelium.

3.2 BBB: PHYSICAL AND MOLECULAR PROPERTIES

The restricted paracellular permeability is due to the combination of physical, molecular barrier properties and specific transporters to deliver specific nutrients.

3.2.1 Physical properties:

There are cell to cell molecular binding systems, adherens junctions (AJ) and tight junctions (TJ). The high electrical-resistant tight junctions only allow small lipid-soluble molecules to cross the BBB. Also, the low rate transcytosis helps to establish this property.

- Adherens junctions:

AJs are composed by vascular endothelium (VE), cadherin, actinin and catenin. Adhesion with adjacent cells is produced through interactions between cytoplasmic extreme of transmembrane VE -cadherin and β-catenin of the adjacent cell, via interaction with α-catenin and Afadin-6, mediated by the binding of actin and catenin to the cytoskeleton (73,74).

- Tight Junctions:

TJs are the main responsible for maintaining limited the paracellular permeability and form the principal physical components of the BBB (65). TJ are a dynamic complex of multiple protein constituents that include a variety of transmembrane proteins such as junctional adhesion molecules, occludins, claudins and membrane-associated guanylate kinase - like proteins (e .g. ZO-1, -2, -3) (75).

TJs provide a high trans-endothelial electrical resistance (TEER) across the BBB (1500-2000 omhios/cm2) that restrict free low of ions and solutes (76).

- Transcytosis:

The rate of transcytosis is very low, in comparison to the endothelium found in other organs, the cerebral microvasculature lacks fenestration and possess only a small number of pinocytic vesicles (77).

There is fluid-phase endocytosis for passing macromolecules and it is induced under pathologic conditions such as ischemic stroke and acute hypertension. Receptor-mediated endocytosis is mediated by caveolin-based vesicle trafficking, triggering internalization of the receptor-ligand complex (78–80).

3.2.2 Molecular properties:

Oxygen, carbon dioxide and small lipophilic substances go freely the BBB but it is impermeable to hydrophilic molecules such as glucose, amino-acid and other nutrients indispensable to life. Furthermore, BBB transport is necessary for the reuptake of neurotransmitters (45).

There are specific transporters expressed in these cells, the efflux transporters which transport lipophilic molecules and highly specific nutrient transporters for specific nutrients or specific waste molecules.

CNS ECs have higher amounts of mitochondria to generate ATP and also express very low level of leukocyte adhesion molecules (LAM) restricting the passage of immune cells to the CNS (81).

- Efflux transporters:

They are situated on the luminal surface and transport lipophilic substrates using the hydrolysis of ATP. Among these transporters are P glycoprotein or multidrug resistance protein 1 (MDR-1), Breast Cancer Resistance Protein (BCRP) and Multidrug Resistance Proteins (MRP). MDR-1 mediates the removal of toxic lipophilic metabolites and cationic drugs; the up-regulation of these receptors has been associated with drug-resistant epilepsy and tumor (82).

- Nutrient transporters:

ECs express a wide variety of these transporters to deliver nutrients from the blood to the brain, many of these belong to the solute carrier class of facilitated transporters, including SLC2A1 gene for producing a protein called the glucose transporter protein type 1 (GLUT1), SLC16a1 for lactate and pyruvate and SLC 7a5 for neutral amino acids, L-DOPA) (77,83).

On the other hand several transports are important for removing waste products from the brain such as RAGE for Aβ (84).

3.3 BASAL MEMBRANE AND EXTRACELLULAR MATRIX

Basal membrane is also called basal lamina, is a thin and uniform structure and is an anchor for ECs and for many signaling processes at the vasculature. BM contains type IV collagens, laminin, heparin sulfate proteoglycans and other glycoproteins. The vascular and parenchymal BMs have a different composition (85). With ageing increases the thickness and are more rigid because the concentration of laminin decreases and concentration of collagen IV increases (86).

EM of the basal lamina is the anchor for the endothelium; this function is mediated essentially by integrin receptors and laminin. There is also evidence that matrix proteins are in maintaining TJ structure (87).

Disruption of these BMs by matrix metalloproteinases (MMPs) is an important component of BBB dysfunction and leukocyte infiltration (88).

3.4 MATRIX METALLOPROTEINASES

MMPs are a family of 26 extracellular and intracellular enzymes with protease activity. Identified as matrix- degrading enzymes, they are involved in the regulation of the extracellular components with processes of opening the BBB. They are implicated in different functions in the body such as development, tumor growth, angiogenesis, response to infection, inflammation, ischemia, injury or fibrosis (89).

MMPs increase the permeability of the BBB acting firstly in EM and secondly in cell signaling and cell death. Their functions are regulated by tissue inhibitors of matrix metalloproteinases (TIMPs) (90). Molecular cascade during neuroinflammation and ischemia, are initiated with the objective of removing damaged cells and repair the tissue. This dual role is constituted by an initial phase with the activation of the process of EM dissembling, opening the BBB and initiating cell apoptosis. In the second phase processes of angiogenesis and neurogenesis are activated (91).

MMPs have a number of important roles in normal development, but they are highly destructive in inflammation of the CNS. MMPs damage the EM, basal lamina and tight junctions in ECs, leading to an increase of permeability of BBB. There are several studies that involve MMPs in processes of neuroinflammatory response in hypoxia-ischemia, multiple sclerosis and infection. But also in process of chronic neurodegeneration associated with vascular cognitive impairment, AD and Parkinson (92).

They have a characteristic molecular structure (93) **[Figure 4].** MMPs are constituted by four domains, a pro-peptide, a catalytic, haemopexin-like and transmembrane domain. The catalytic domain contains fibronectin-binding sites and the zinc and calcium atoms. They are zinc and calcium-dependent endopeptidases. Hemopexins are joined by a hinge region and are attached to a transmembrane domain.

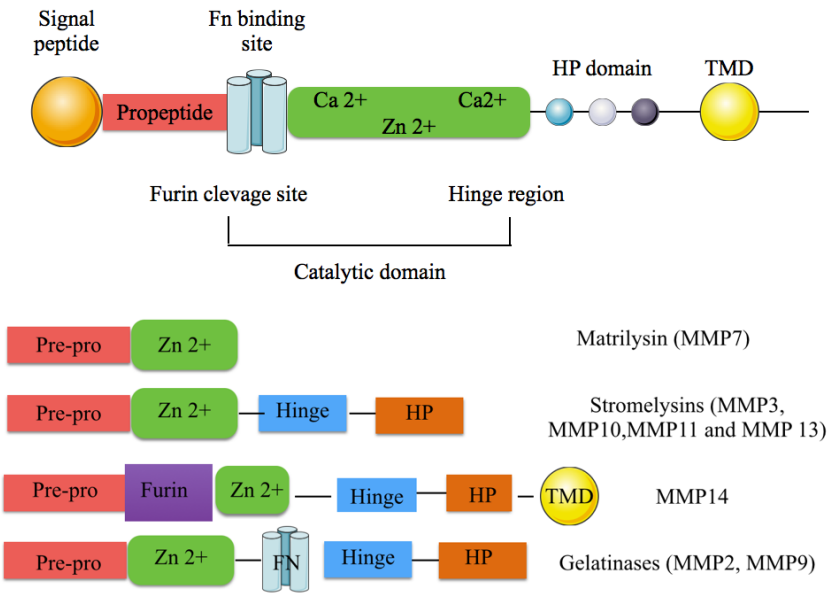


Figure 4: MMPs protein structure. A signal peptide and the propeptide region form part of the cysteine switch, which folds over the zinc in the catalytic site and maintains a latent state. A cleavage site enables the proconvertase furin to activate MMP by cleaving the propeptide. An FN binding site is present in MMP2 and MMP9, connecting them with the basal lamina. The catalytic site is present in all MMPs. A haemopexin domain is joined to the catalytic site by the hinge region. MMP 14 has a TMD. Own figure modified from (93).

Characteristically, MMPs are synthesized in a latent way. The signal peptide and the pro-peptide segment that maintains the latency state of the enzyme are located at the N-terminus. The pro-peptide is activated by the dissociation of cysteine. The exposure of the catalytic site is named the cysteine switch (94).

MMPs are synthesized as latent form, as inactive zymogen and are secreted into the extracellular space. Pro-MMPs are activated by disruption of the zinc-thiol interaction between the catalytic site and the pro-domain (95).

MMPs are divided into four main subgroups **[Table 1]**:

1. Collagenases (Collagenase-1 or MMP-1), degrade fibrillar collagens and mainly are in bone and cartilage.

2. Gelatinases (MMP2 and MMP9) have important activity in the brain. They have fibronectin structure (FN) and haemopexin domain (HP) that is joined to the catalytic site by the hinge region, but lack the furin-binding region.

3. Stromelysins: include Stromelysin-1 (MMP3) and Matrilysin (MMP7, MMP26) and MMP8, MMP10, MMP 11, MMP12 and MMP13. Matrilysin has not a hemopexin domain and it is the smallest of MMPs.

4. Membrane-type MMP: MMP14 has a transmembrane domain (TMD) and it is also known as MT-MMP1 (96).

Classes	MMP	Substrate
Interstitial collagenases	Collagenase-1 (MMP-1)	Collagen I, II, III, VII, X aggrecan, serpins
Stromelysins	Stromelysin-1 (MMP-3)	Collagen IV, V, IX, fibronectin, elastin, laminin, aggrecan, fibrillin, osteonectin
	Matrilysin (MMP-7)	Elastin, fibronectin, laminin, nidrogen, collagen IV,
Gelatinases	Gelatinase A (MMP-2)	Collagen I, II, V, VII, X, elastin, fibrillin, osteonectin
	Gelatinase B (MMP-9)	Collagen IV, V, VII, X, XIV, elastin, fibrillin, osteonectin
Membrane-type MMP	MT1-MMP (MMP-14)	Collagen I,II,III,IV, laminin, aggrecan, tenascin, nidrogen, fibrillin, fibrin.

Table 1. Main classes of the Matrix Metalloproteinases. Modified from (97)

The proteins remain latent or inactive until they are activated by free radicals or enzymes that free the cysteine bond or cleave the pro-peptide region. Most of MMPs , with the exception of membrane-type MMPs (MT-MMPs) are secreted and act in the extracellular space. MT-MMPs contain a furin cleavage site near the pro-peptide region and are

activated intracellularly by the proconvertase furin, and the serin protease plasmin (98).

MMP-14 (or MT1-MMP) has an NF-kB binding site, suggesting that can also be induced during inflammation.

There are two types of MMPs in the brain, constitutive and inducible proteases. Constitutive enzymes such as gelatinase A (MMP-2) and membrane type-1 metalloproteinase (MMP-14) are present all the time and have functions of maintaining normal extracellular matrix; these constitutive MMPs are present in latent forms, and become active in hypoxic/ischemic injury. MMP-2 comes from the activation of proMMP-2 by the action of MMP-14 and tissue inhibitor of metalloproteinases-2 (TIMP-2). MMP-2 is normally present in brain tissue and in the CSF. Gelatinase-B (MMP-9) attacks similar substrates but is normally only present at low levels and is markedly upregulated in inflammation (99,100).

Two other enzyme families, a disintegrin and metalloproteinase (ADAM), a disintegrin and metalloproteinase domain thrombospondin (ADAMTS) and serine proteases, plasminogen/plasminogen activator system, are also involved in extracellular matrix degradation (101).

Inducible enzymes such as MMP-3 and MMP-9 are induced by inflammatory cells, including microglia/macrophages, after injury. In the case of MMP9, several activation mechanisms have been suggested, such as other proteases (e.g. MMP3) and free radicals (e. g. nitric oxide). Inducible enzymes are more destructive since they are released into the extracellular space. This start of inducible MMPs causes irreversible damage while the initial phase is still reversible (99).

TIMPs are enzymes with inhibitory actions against MMPs (TIMP-1 to TIMP-4); TIMP1 that mainly inhibits MMP9 and TIMP2 that inhibit MMP2, and paradoxically contribute to activation of pro-MMP2; both are located into the EM. The expression of TIMPs in the tissue is regulated during tissue remodeling and physiological conditions to maintain a balance in the metabolism of the EM (102). TIMP-3 is the only TIMP to inhibit a non-matrix type metalloproteases like tumor necrosis factor alpha (TNF α) converting enzyme (TACE) and an inhibitor of MMP-3 and MMP-7 (103).

	FUNCTION	INHIBITOR
Gelatinases (MMP-2 and MMP-9	Cause disruption of the blood-brain-barrier, angiogenesis, neurogenesis, remodeling of the basal lamina, regeneration of axons, remyelination, and apoptosis	All TIMPs
Stromelysins (MMP-3 and MMP-10) Matrilysin (MMP-7)	Cause proteolysis of proteins in the extracellular matrix, disruption of the blood-brain barrier, angiogenesis, synaptic remodeling, glutamate receptor proteolysis, and apoptosis.	All TIMPs
MT-MMPs (MMP14, also known as MT1-MMP)	From trimolecular complex with TIMP2 and pro-MMP2 for activation of MMP2 at cell surface	TIMP3
ADAM10	Acts as alpha secretase in amyloid precursor protein proteolysis, degrades NOTCH, acts as sheddase at cell surface for growth factors and integrins	TIMP1 TIMP3
ADAM17 (TACE)	Acts as alpha secretase and sheddase for TNF alpha receptors at the cell surface	TIMP3

Table 2. Main functions of the Matrix Metalloproteinases. Modified from (104)

3.4.1 MMPs in vascular cognitive impairment (VCI)

There are multiple references in relation to the role of MMPs in small vessels disease and vascular cognitive impairment. In tissue of brains affected by Binswanger disease (arteriosclerosis of the blood vessels and demyelination of the white matter), macrophages were stained for MMP-3 (93). Also in peripherally blood MMP-2 are associated with previous volume of leukoaraiosis in acute stroke patients in patients with small vessel disease (105).

Vascular risk factors (hypertension, hyperlipidemia, diabetes, advanced age) also have been implicated in the increment of the function of these enzymes causing the permeability of the BBB (106).

In relation to the possible mechanism for white matter injury in VCI , one hypothesis is that hypertension, diabetes mellitus and other diseases that damage blood vessels can initiate white matter injury by

increasing the hypoperfusion in the watershed area of the deep white matter (107–109).

Astrocyte cultures when are stimulated by Il 1-β or TNF α secrete pro-MMP2 and proMMP-9. One hypothesis is that hypoxia secondary to hypoperfusion increases HIF α that leads to activation of furin, that is an activator of MT1-MMP that induces activation of MMP2 that breaks the tight junctions. Also MMP2 provokes the activation of endothelin-1 that causes vasoconstriction (110).

MMP-2 initiates the process to break fibronectin and laminins initiating the BBB breakdown; as hypoxia and inflammation progressed the inducible stromelysin-1 (MMP-3) and gelatinasa B (MMP-9) are up regulated producing increasing of blood-brain barrier injury and neuronal death, including demyelination and oligodendrocyte damage; this term is known by-stander demyelination and it is not an immunological process (111).

ProMMP-9 is activated by MMP-3 (99). ECs have mainly MMP-9, pericytes express MMP-3 and -9, while astrocytes have MMP-2 and MT1-MMP. This pattern of MMPs facilitates the opening of the BBB in ischemia or hypoxia, but also allows for the gradual changes in the EM (99).

Additionally, MMPs are implicated in mechanism of repair with physiological processes. HIF α are activated mediated by the expression of VEGF and TGF β (104).

MMP2, MMP3 and MMP9 have roles in angiogenesis and neurogenesis. MMP9 has roles in remyelination, as oligodendrocyte regrowth (112) and promote the movement and migration of neural progenitor cells to the injury site, like neuroblasts located in subventricular zone (113).

MMPs are triggered also by a variety of stresses, not only hypoxia, but also oxidative stress and osmotic shock (114).

3.4.2 MMPs in Alzheimer disease:

MMPs are involved in neurodegenerative processes such as the formation and clearance of the Aβ peptides in AD. MMPs are induced endogenously by the amyloid molecules in blood vessels, astrocytes

and microglia. Astrocytes exposed to Aβ 1-40 secrete MMP2, MMP3 and MMP9 (115). This inflammatory response contributes to neuronal death.

MMP-2 and MMP-9 are implicated in the physiological degradation of Alzheimer´s Aβ and are associated with vascular amyloid deposits. Plasma concentrations of MMP-9 are increased in AD, MMP-9 can catabolize the fibrillary form of Aβ and can contribute to the elimination of soluble and fibrillary forms (116). MMP2 also contributes to degradation of Aβ 1-40 and clearance of this products into the blood.

MMP-9 was implicated in CAA related ICH, vessels affected by ACC expressed extensive MMP-9 immunoreactivity, suggesting a role in the pathogenesis of spontaneous intracerebral hemorrhage (117).

MMP-9 stimulated by Aβ induces extracellular matrix degradation **[Figure 4].** In brains of patients affected by ICH, MMP-2 and MMP-9 reactivity were found perihematoma, in vessels damaged by CAA and in chronic MHs (118).

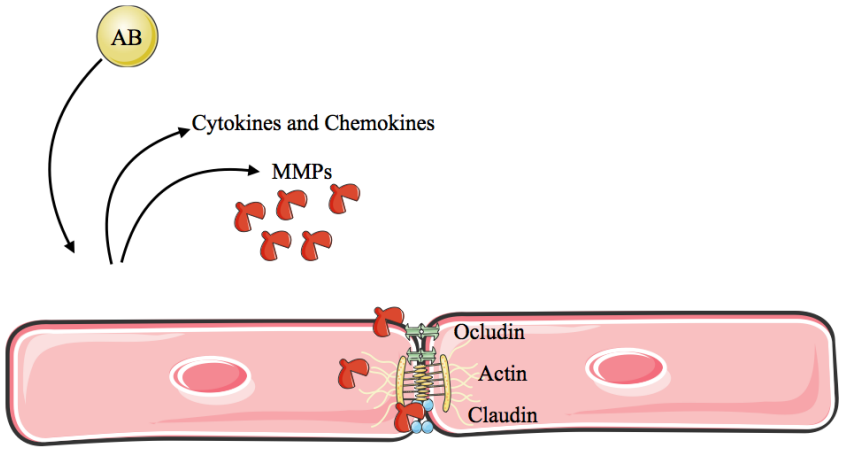


Figure 4: Possible mechanisms leading to BBB disruption in presence of amyloid β. Own figure, modified from (119)

The effect of inhibitors of metalloproteases in acute processes such as stroke, ICH, autoimmune or infectious disease has been studied with favorable results (120–124).

But in chronic processes such as AD and VaD is more complicated with important side-effects, since these molecules have double effects with capacity for regeneration and also for the removal of Aβ.

3.5. ASTROCYTES

Astrocytes are the most abundant glial population in CNS; their extended terminal processes that ensheath either neuronal processes such as synapsis or blood vessels. The end-feet of the vessels is form by dystroglycan, dystrophin and aquaporin 4. The dystroglycan-

dystrofin that links cytoskeleton with BM is important to bind the end-feet to BM (125).

Astrocytes have an important role in the regulation of water homeostasis of the brain through the function of Aquaporin-4 (AQP-4) **[Figure 5]** (126).

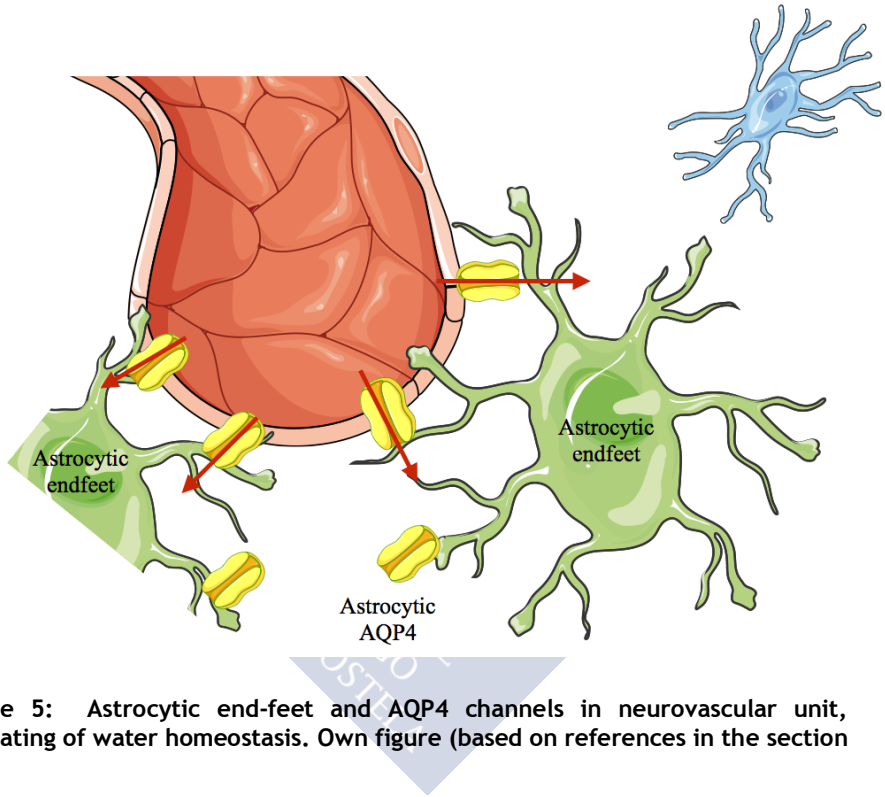


Figure 5: Astrocytic end-feet and AQP4 channels in neurovascular unit, regulating of water homeostasis. Own figure (based on references in the section 3.5)

AQP4 intervenes in the regulation of extracellular space volume, the CSF circulation, the interstitial space fluid (ISF) resorption and the elimination of waste products of the neurons metabolism. Also functions in potassium homeostasis (favoring clearance of K), cell excitability, synaptic plasticity, neuroinflammation and cell migration were described (127).

Aquaporin are water channels located in the membranes of several cells in the organism, and were described in the 90s. AQP4 was

described in brain specifically, and located in ependymal cells and astrocytes; they are situated polarized in greater number in the regions that cover capillaries in perivascular glial end-foot membrane or pia surface membrane in contact with CSF (128,129).

Astrocytes play a key role in the regulation of some essential functions such as the blood flow control by regulating the contraction/dilation of vascular smooth muscle. Astrocytes produce the angiotensin converting enzyme-1 (ACE-1), which converts angiotensin I into angiotensin II (AT2) that induces vessel contraction, AT1 restricts BBB permeability and stabilizes junctional protein function. Though AT 2 signaling to astrocytes plays an important role in the regulation of leukocyte infiltration to the CNS in response to neurodegenerative stimuli (130,131).

Moreover they regulate the physical permeability and trans-endothelial electrical resistance (TEER) enhancing tight junctions using Hedgehog (Hh) signaling cascade that induces the expression of junctional proteins (132).

Astrocytes secrete Transforming Growth Factor beta (TGF- beta) which is a cytokine implicated in cell growth, differentiation, morphogenesis, apoptosis and immunomodulation. TGF in CNS is neuro-protective being that up regulates the tight junction and down regulates the leukocyte migration through the endothelium. (133)

Also, certain MMPs are secreted by astrocytes too.

Additionally they have an important contribution to the maintenance of the cerebral ion homeostasis, (134) and serve as an interconnection with subcortical neurons and interneurons (52).

3.6 PERICYTES

Pericytes (PCs) are in CNS in a greater proportion than in the rest of the organs and have several functions, using multiple signaling pathways; pericytes have a role in the stability, in the modification of the permeability of the BBB, including the formation of TJs and vesicle trafficking in CNS endothelial cells.

PCs do not induce BBB-specific gene expression in CNS endothelial cells, but inhibit the expression of molecules that increase

vascular permeability and CNS immune cell infiltration; in particular, PCs down regulate the rate of transcytosis and the expression of LAMs (135). They also have functions in angiogenesis (136).

3.7. MICROGLIA

Two main immune cell populations, macrophages and microglial cells, are not a basic elements of the BBB but have an impact on BBB function and integrity. Upon any detection of signs for brain lesions or nervous system dysfunction, microglial cells undergo a complex, multistage activation process that converts them into the "activated microglial cell¨ (137). Also macrophages are commonly found in VRS; these cells provide a first line of innate immunity by phagocytizing cellular debris (138).

Leukocyte migration is produced through a set of surface adhesion molecules of leukocytes and vascular endothelial cells. Tethering and rolling of leukocytes are achieved via integrins such as VLA-4, and adhesion molecules such as ICAM-1, VCAM-1 and PECAM-1. This process is usually initiated by cytokines that activate the endothelium (139).

3.8 NEURONS

There is a direct innervation by noradrenergic, serotoninergic, cholinergic and gabaergic neurons on the astrocyte but also on the BBB directly and they have influence on behavior of capillary endothelial barrier (140).

4. CONTROL OF CEREBRAL MICROCIRCULATION

4.1 CEREBRAL HEMODYNAMICS

The brain receives 20% of the total blood flow and since it mainly uses glucose for its energy metabolism has to maintain a constant blood supply in order to maintain a cerebral perfusion with minimal variability (141).

Cerebral blood flow (CBF) is driven by cerebral perfusion pressure (CPP), represented by the difference between mean arterial blood pressure (ABP) and intracranial pressure (ICP). These components working together with cerebrovascular impedance, including cerebrovascular resistance, which is inversely proportional to the forth power of the vessel radius. The radius is the main determinant of blood flow and even small changes in diameter have significant effects on cerebral blood flow (142).

Other important component is the internal fluid resistance which depends on viscoelastic properties of arterials walls, vascular compliance, elasticity of the vessel wall, viscosity of the blood and flow velocity (143).

4.2 AUTOREGULATION OF CEREBRAL BLOOD FLOW

Autoregulation is the ability of the brain to maintain relatively constant blood flow despite changes in perfusion pressure. It is imperative in brain due to the need of a constant blood supply and water homeostasis.

CBF is maintained at around 50 ml per 100 g of brain tissue per minute, providing that CPP is in the range of approximately 60-160 mmHg, in normotensive adults (144).

Exceeded these limits above or below, brain auto-regulation is lost and CBF becomes dependent on mean arterial pressure in a linear fashion. When CPP falls below the lower limit of autoregulation cerebral ischemia appears (142).

Mechanisms of autoregulation are not completely understood but according to the results of several experiments there are three different control pathways: vasogenic, metabolic and neuromediator (143).

4.3 VASOGENIC MECHANISM

Vasogenic mechanism is related to myogenic response which is an intrinsic property of smooth muscle to response to changes in intravascular pressure and shear stress (145).

The smooth muscle constricts in response to an increased pressure and dilates in response to a decreased pressure. Mechanism is mediated by the depolarization of the smooth muscle cell membrane in response to increased pressure: this depolarization causes calcium influx via of voltage-operated calcium channels opening (CaV). Intracellular calcium increases myosin light-chain kinase which phosphorylates myosin light chain and causes muscle contraction (146).

Shear stress-induced vasodilatation is produced through the interaction between the endothelial cells which produce nitric oxide (NO) due to the increase in endothelial NO synthase (eNOS) activity (147). NO synthase (NOS) is the enzyme responsible for the oxygen-dependent conversion of L-arginine to NO and L- citrulline. There are three isoforms in brain: neural (nNOS), endothelial (eNOS) and inducible (iNOS): this last one is not normally expressed in brain, but its expression can be induced under pathological conditions such as ischemia, reperfusion, hypertension, diabetes or subarachnoid haemorrhage (148,149).

Activation of nNOS and eNOS requires Ca2+/calmodulin, hence NO production from these two isoforms is initiated and modulated by elevated intracellular free Ca2. Tetrahydrobiopterin (BH4) is a requisite cofactor for this process (150).

NO diffuses into the smooth muscle producing vasodilation by the activation of potassium channels that produce cell hyperpolarization (151). This mechanism is mediated by the activation of soluble guanylyl cyclase (SGC) in smooth muscle cells: the activation of SGC increases the levels of cycling guanine monophosphate (cGMP)

causing the opening of calcium-activated potassium channels (BK Ca) (152).

In addition to synthesizing NO, purified nNOS catalyzes superoxide under situations related to decreased L-arginine or tetrahydrobiopterin (BH4) due to oxidation or reduced formation and superoxide mediated cell injury (153).

Endothelium can produce other molecules with vasodilator power such as endothelium-derived hyperpolarizing factor (EDHF) which diffuses to smooth muscle cells and activates smooth muscle Potassium channels (Kcs) causing hyperpolarization and closure of Ca V channels and relaxation (154).

Other products of metabolism of arachidonic acid (AA) in the endothelial cells such eicosanoids like Prostaglandin I2, E2, D2 causing vasodilation; however from the AA cascade can also be derived constricting metabolites like Tromboxano A2 or prostaglandin F2 alpha (155).

4.4 METABOLIC REGULATION

In metabolic regulation arterial resistance is modified by CO2 tension in periarteriolar space but not in a direct way because hypocapnia or hypercapnia results in alterations in the extracellular pH; hypocapnia leads to cerebral vasoconstriction and hypercapnia leads to vasodilatation. Hydrogen ions may directly activate Kcs in smooth muscle cells leading to hyperpolarization and vasodilation (156).

Hypoxia, potassium ions and adenosine also leads to hyperpolarization (157).

4.5 NEURAL-ASTROCYTE REGULATION

The neurovascular coupling between blood flow and neuronal activity to meet the metabolic demand in one area of the brain and get the blood needed (functional hyperemia) is dependent on astrocytes and perivascular neurons, and neuron to astrocytic signaling is essential to the dynamic control of brain circulation (158).

Increased local neural activity leads to a release of glutamate acting on astrocytic metabotropic glutamate receptors, increasing the synthesis of arachidonic acid which is metabolized to vasodilatory prostanoids like PGE2 (159); it also leads to the activation of neuronal NO synthase and production of NO on perivascular neurons which acts as a modulator of this process (160).

4.6 IMPAIRMENT OF CEREBRAL HEMODYNAMICS IN SVD

In the case of SVD there is a loss of CBF autoregulation due to an increase in peripheral resistance and, as consequently, a higher sensitivity for hypoperfusion. (161) Endothelial dysfunction, rupture of BBB and deposit of collagen and Aβ and arterial stiffness are the main mechanisms to produce this process of alteration of the microcirculation.

We can study the hemodynamics of SVD trough ultrasound. Transcranial and carotid doppler ultrasonography are techniques used to assess cerebral microcirculation using static and dynamics methods to measure of blood flow velocity changes in cerebral arteries (162).

The pulsatility index (PI) evaluates the relationship between cardiac function and peripheral resistance. The resistance index (RI) is similar, since it reflects the resistance to arterial flow originated by the distal microvascular bed distal to the measurement site. In territories with high resistance of the distal vessels a low diastolic flow is produced in the artery responsible for giving irrigation to that area; there will be a marked difference between systolic velocity peak and the final diastolic velocity.

Studies of cerebral hemodynamics in leukoaraiosis have found an association between WMH volume and increased pulsatility and RI values. Moreover, they have found it is related with aging and in patients with dementia (163,164).

Carotid ultrasound permits the assessment of intima media thickness (IMT), carotid stenosis, atheroma plaque burden and unstable plaques. Carotid IMT is defined as the maximum distance between the characteristic echoes from the lumen-intima and media-adventitia interfaces on the far wall of the common carotid artery. It is a biomarker

of subclinical atherosclerosis and endothelial dysfunction (165). Carotid plaques are defined as focal structure that encroaches into the arterial lumen of at least 0,5 mm or 50% of the surrounding IMT value or demonstrates a thickness > 1,5 mm (166).

There are several studies about the relation between increase in carotid diameter, carotid PI and RI in patients with WMH with hypertension (167). Also plaque instability and IMT and plaque burden are correlated with WMH lesions and mild cognitive impairment (168,169).

5. CLEARANCE PATHWAYS AND PERIVASCULAR SPACES

The circulation of CSF includes its formation and its elimination towards the circulatory and lymphatic system. A large part of CSF is produced by the choroid plexus, formed by a net of capillaries comprised by a specialized endothelium and covered by choroid epithelium located in the ventricles. This system is one of those used for the elimination of waste substances from the cellular metabolism of the brain (170).

Clearance or removing of waste products from the cerebral cellular metabolism occurs via various overlapping systems. These systems are classified according to the compartment from which the product is discarded and the compartment that receives it. Protein waste can be cleared intracellularly due to the activity of enzymes like ubiquitin proteasome, through lysosome activity by endosomes or autophagy lysosomes or extracellularly by proteases or glial phagocytes. But waste proteins can be exported into the blood or lymph, or recirculated in the CSF (171).

Several waste protein removal systems have been identified through studies. The first one was performed by Cserr and collages and carried out in mouse animals models by radioiodinated albumin injection in the CSF and in the extracellular space; they observed how

the radioactive serum album was drained to cervical lymph nodes and deposited in leptomeningeal artery walls (23).
We can distinguish clearance trough the BBB with a flow from the ISF to the blood; this system is believed to be the one that predominates in waste products clearance. Transporters expression such as low density lipoprotein receptor-related protein (LRP) 1, LRP2, ATP-binding cassette sub-family B member 1 (ABCB1), Receptor for advanced glycation end products (RAGE) and Apolipoprotein E (ApoE), alpha 2 macroglobulin (alpha 2M) mediators with activity of influx or efflux affect the function of this system mainly in the case of Aβ (172–174).

The products that are discarded through the flow of ISF could be directed towards CSF directly, or towards peripheral lymph by perivascular spaces **[Figure 6]** (175).

Perivascular spaces (PVSs) are channels surrounding cerebral arteries and veins, also known for years by Virchow Robin spaces (VRSs) that it is a histopathological term. VRS drive circulation of ISF and CSF towards the lymph. (176). The perivascular drainage moves waste into the periarterial space, located along smooth cells and capillary basement, and towards the subarachnoid space in the opposite direction to blood flow. Subarachnoid CSF enters the brain parenchyma via perivascular channels of the penetrating arteries, which surrounds arterioles and venules and leading the waste products to the perivenous glymphatic **[Figure 7]** (177).

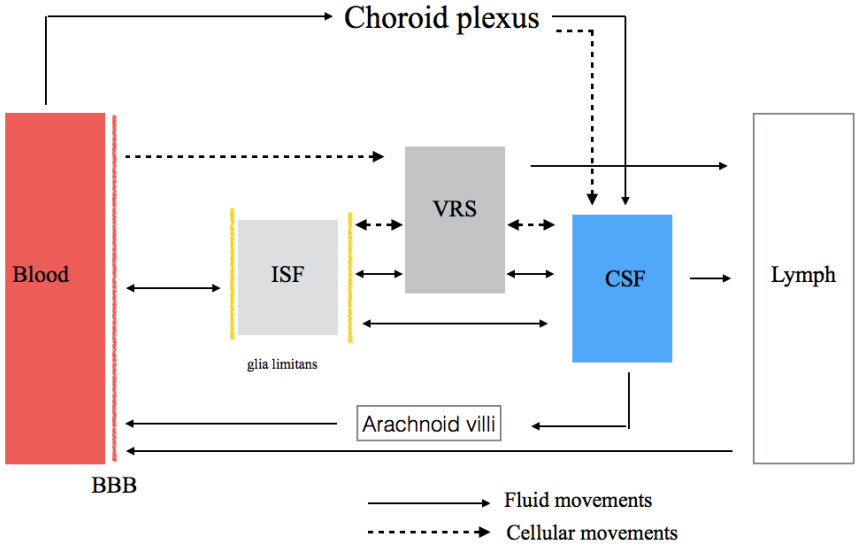


Figure 6: Diagram of cerebrospinal fluid circulation: fluid and cellular movements across the different barriers of the brain compartments (blood, interstitial fluid, Wirchow Robin space, cerebrospinal fluid space comprising the cerebral ventricles, basal cisterns and cortical subarachnoid space). Own figure, modified from (171)

In this route of waste products elimination, the role of ApoE and Aquaporin 4 (AQP4) expression, in the case of Aβ, is very important. Aβ clearance is reduced in AQP4 knockout mice (178); AQP4 is a water channel expressed in perivascular astrocytic endfeet where water components of the CSF cross the BBB to enter the brain parenchyma **[Figure 8]** (178)

The presence of ApoE4 is associated with reduced perivascular drainage of Aβ. (179)

Regarding the elimination of waste products, in the brain there is not a conventional lymphatic system, however, the presence of lymphatic structures was reported and are located next to dural sinuses.

These lymphatic vessels transport CSF and ISF from subarachnoid spaces and brain to the cervical lymph nodes. (180)

The interstitial fluid can be cleared directly into CSF through CSF sink (subarachnoid space or through ventricles) or through the perivascular space pathways. These perivascular space have two ways of circulation, one in which CSF travels in the direction of the blood and another in which the ISF goes towards the subarachnoid space in the opposite direction to the circulation of the blood (174,181).

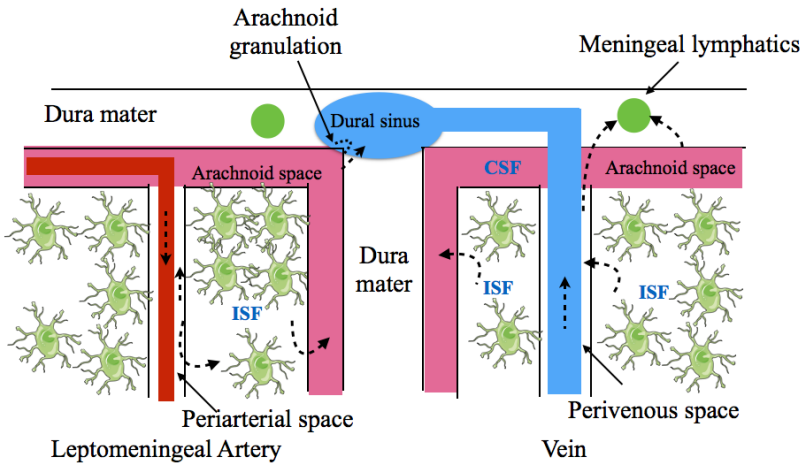


Figure 7: Perivascular clearance: perivascular clearance comprises perivascular drainage and glymphatic pathways. The perivascular drainage moves waste into the periarterial space and towards the subarachnoid space in the opposite direction to blood flow. Perivenous space moves waste towards meningeal lymphatics. *Own figure, modified from* (182)

The perivascular drainage is dependent also on arterial pulsations from vascular smooth muscle cell contractions, respiration, sleep and CSF pressure gradients (183).

With aging, neurodegeneration, and cerebrovascular disease, these microscopic PVSs can become enlarged known as enlarged perivascular spaces (EPVS) allowing their visualization by MRI.

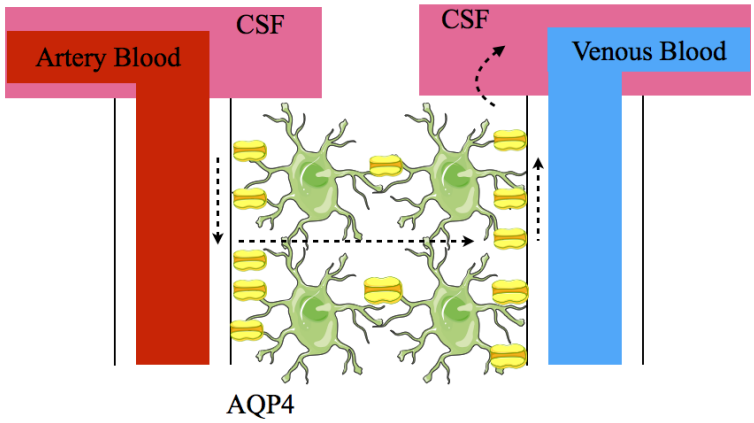


Figure 8: Perivascular spaces and AQP4 channels: water components of the CSF crosses the BBB to enter the brain parenchyma.
Own figure, modified from (184)

CSF can be absorbed directly into the circulatory or lymphatic systems. The plexus choroid serves also as ventricular solute clearance site, CSF in the subarachnoid space could be cleared from the brain at arachnoid villi towards the dural venous sinuses. CSF can be cleared from brain to cervical lymph nodes by perivascular space, but another route is through the perineural spaces, extensions of the subarachnoid space surrounding the nerves (185,186).

There are evidences that the decrease in Aβ peptides clearance into the CSF leads to deposition in vessels inducing CAA and interfering with perivascular drainage. This becomes clear on neuroimaging by an enlargement of the perivascular spaces (42,187,188).

6. CEREBRAL MICROANGIOPATHY

6.1 DEFINITION

SVD or cerebral microangiopathy (CM) refers to a group of diseases with various etiologies that affect the small arteries, arterioles, venules, and capillaries of the brain and the resulting brain damage in the cerebral white and deep grey matter. The definition of a small vessel is not consistent, but the current definition refers to all vascular structures (ranging from around 5 micro-millimeters to 2 millimeters) in the brain parenchyma or the subarachnoid space (47) **[Figure 9]**

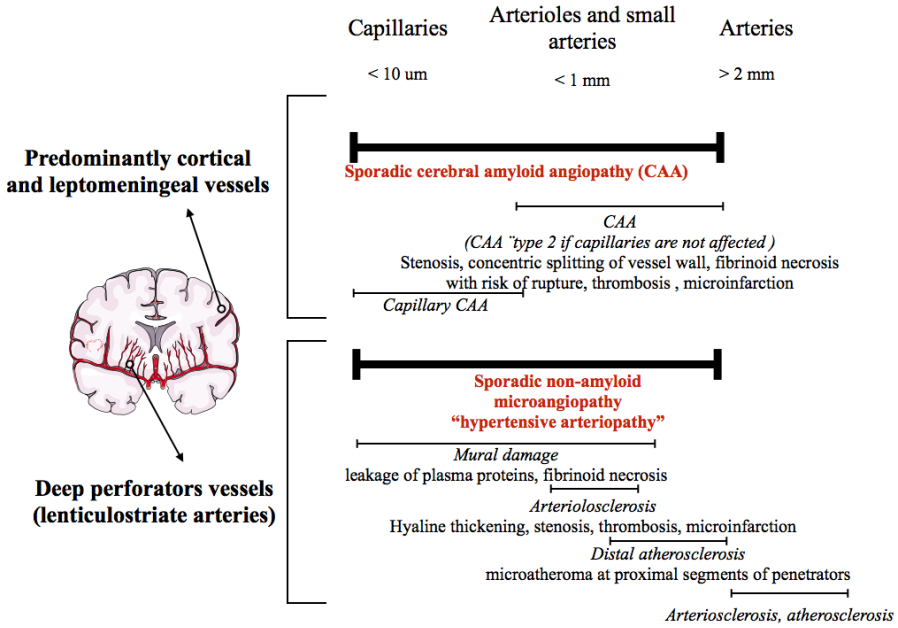


Figure 9: Scheme of main forms of SVD sporadic amyloid angiopathy and hypertensive angiopathy. Own figure, modified from (4).

The term is commonly used to describe a range of neuroimaging, neuropathological, and associated clinical features. The brain parenchymal MRI-lesions they are thought to cause have been adopted as markers of disease involving small vessels, as a result, the term SVD is frequently used indiscriminately to describe both the underlying small vessel pathologies and the neuroimaging correlates.

Terminology for clinical features, imaging and the pathology of SVD is highly varied. Standardization of terminology for imaging features was illustrated in 2013 in an international working group position paper from the Centers of Excellence in Neurodegeneration under the acronym Standards for Reporting Vascular changes of nEuroimaging (STRIVE) (36).

6.2 CLASSIFICATION: FORMS OF CEREBRAL MICROANGIOPATHY

There are different types of small vessel diseases, the most frequent form is sporadic SVD which includes sporadic amyloid angiopathy and sporadic non-amyloid angiopathy (4) traditionally also known as hypertensive arteriopathy, arterioloesclerosis, age related or vascular-risk-factor related or degenerative microangiopathy in the literature (1)

Other SVD are inflammatory and immunologically-mediated small vessel diseases such as vasculitis, which are a heterogeneous group of rare diseases characterized by the presence of inflammatory cells in the vessels (for example Wegener´s granulomatosis, Churg-Strauss syndrome, microscopic polyangiitis, nervous system vasculitis secondary to infections or associated with connective tissue disorders). There are also genetic forms of SVD distinct from CAA. For instance, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) , cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL), Fabry´s disease, SVD caused by COL4A1 mutations, hereditary cerebroretinal vasculopathy (CRV), hereditary vascular retinopathy (HVR), Hereditary endotheliopathy with retinopathy nephropathy and stroke (HERNS) hereditary multi-infarct dementia of

the Swedish type and mitochondrial encephalomyopathy, Lactic Acidosis and Stroke-like Episodes (MELAS).

Other forms less frequent are post-radiation angiopathy and venous collagenosis (4).

7. SPORADIC AMYLOID MICROANGIOPATHY

Amyloidosis is a group of diverse disorders caused by abnormal folding and extracellular deposition of proteins as insoluble amyloid fibrils. Deposits cause a tissue damage and organ dysfunction, especially in brain and nerves, heart and kidneys (189).

Amyloidosis is classified as localized and systemic. In the localized subtype, amyloid is deposited in the organ or tissue of the precursor production and amyloid deposition is confined to a certain organ or tissue. In the systemic subtype, amyloid is deposited in one or more sites distant from the site of the precursor production (190).

CAA is classified according to the amyloid protein deposit involved, including Aβ , Cystatin C, Prion protein (PrP), Transthyretin (TTR), Gelsolin, ABri/ADan (in familial British dementia and familial Danish dementia) and immunoglobulin light chain (AL) (191,192).

The most frequent type is sporadic Aβ-type CAA which is commonly found in elderly individuals as well as in patients with AD. Hereditary or genetic forms of Aβ -type CAA includes mutations in the Aβ -protein precursor (Aβ PP) gene, mutations of presenilin genes and Down syndrome (193,194).

7.1 EPIDEMIOLOGY OF SPORADIC AMYLOID MICROANGIOPATHY

The incidence of CAA increases with aged (195). Cerebrovascular amyloid is present in approximately 10-40% of normal elderly brains (196), although it may reach higher prevalence in people older than 90 years (197).

Several studies have suggested an overlap between CAA and AD. Vascular and parenchymal Aβ deposition are usually observed at the same time; CAA is present in nearly 80% of brains with AD and even

moderate to advanced amyloid pathology is present in approximately 25% of brains with AD (198).

CAA increases the risk of spontaneous lobar haemorrhages in the elderly, and is the second most common cause of ICH; the proportion of spontaneous hemorrhages attributable to CAA range to 10-20% in autopsy series (199).

7.2 NEUROPATHOLOGICAL ASPECTS

Vonsattel graded CAA (200) with respect to the severity of pathological changes. Amyloid initially tends to deposit around smooth muscle cells in the abluminal portion of the tunica media and adventitia (mild). This is followed by progressive destruction of smooth muscle cells (moderate) and, subsequently, by degenerative changes such as: disruption of the vessels architecture with hyaline degeneration, vessel wall thickening and reduction of lumen diameter in arteries, even vessel occlusion is produced, "double-barreling", microaneurysm formation and fibrinoid necrosis with evidence of perivascular leakage of blood and weakening of the vessel wall leading to dilation of the lumen (severe) (19,22,201).

There are two pathological types: CAA type 1 with Aβ deposits in cortical capillaries and CAA type 2 with Aβ deposits in leptomeningeal and cortical arteries but not capillaries. AD cases with capillaries involvement are associated with the presence of APOE epsilon 4 allele (202). Moreover, capillary form is most frequent in the occipital lobe, and associated with the severity of CAA and severe AD neuropathology, correlating with the hypothesis of neuronal origin of Aβ via drainage from ISF (203,204).

Perivascular and intramural inflammatory infiltrates are observed with activated microglial cells, lymphocytes and macrophages (205,206).

7. 3 PATHOGENESIS

Aβ -related peptides are secreted by a variety of cell types, but certain peptides and their absolute levels are dependent on cell type. Human astrocytes produce higher levels of Aβ than other cells (207).

Aβ peptide is the product derived by proteolytic processing from the amyloid precursor protein (APP) which is an integral transmembrane glycoprotein (208). It has different isoforms of different molecular weight, the beta segment has 28 amino acids from the outer domain and about 11 to 14 from the transmembrane domain.

Generation of Aβ from Aβ PP requires two proteolytic events, the action of the alpha secretase, that divides the beta segment in two equal parts and then gamma secretase, that divides the transmembrane domain. This way that produce non-pathogenic soluble fragments, it is called the secretory pathway or non-amyloidogenic (209).

The proteolytic process occurs different in affected cells where beta secretase has the predominate activity. The β-site amyloid precursor protein cleaving enzyme (BACE-1) and gamma secretase, which has been recently characterized as part of presenilin complex, producing cleavage of APP resulting in Aβ peptides (210,211). There are different isoform produced as a result of abnormal Aβ production. They are differentiated by the number of C-terminal amino acids. The 40 amino-acid-long (Aβ 1-40) is more soluble than Aβ 1-42, and therefore accumulates in the walls of the vessels , however Aβ 1-42 plays a crucial role in the pathogenesis of AD since it is deposited as insoluble aggregates of oligomers and protofibrils, resulting in fibrillary species accumulating in neuritic and senile plaques (208,212). Aβ 1-38 is produced in smaller amounts (<20%) and has less tendency to deposit, both in the brain and in the blood vessels than the other isoforms.

Aβ protein in CAA is predominately composed of Aβ 1-40 and in smaller proportion of Aβ 1-42, with the Aβ 40/ Aβ 42 ratio higher than in senile plaques (213).

Feature	Cerebrovascular Amyloid Deposition	Senile Plaque Amyloid Deposition
Predominant A type	A 1-40	A 42
Location of A deposition	Occipital lobe predominance	Frontal, parietal, temporal lobes
Cerebral microbleeds	Lobar, predominant occipital	No associated with senile plaques
Location of white matter disease	Equal distribution between anterior and posterior subcortical	Equal distribution between anterior and posterior subcortical

Table 3: Comparison of Features of Cerebrovascular versus Parenchymal Senile Plaque Amyloid Deposition Modified from (214)

The neurotoxic cascade is perpetuated, Aβ oligomers produce activation of proinflammatory cell pathways, mitochondrial dysfunction, increased oxidative stress and tau phosphorylation, disturbance in the regulation of calcium metabolism (215), alteration of the BBB, cell toxicity and apoptosis (216,217).

Structural components of blood vessels (smooth muscle cells, pericytes, endothelial cells) also express Aβ, but is proposed that there is a neuronal origin of the Aβ deposited in the blood vessels of the brain. It has been suggested that Aβ is drained along the perivascular interstitial fluid pathways of the brain parenchyma and leptomeninges, and deposited along the vessels under specific pathologic conditions.

7.3.1 Clearance and perivascular drainage of Aβ: Protein elimination failure angiopathy

The cerebrovascular deposit of amyloid B causes amyloid angiopathy. Aβ 1-40 is deposited in the perivascular pathways in the basal lamina of arterioles and capillaries of the leptomeningeal cerebral cortex.

There is a consolidated evidence that AAC is a protein elimination failure angiopathy (PEFA) (43,218,219). This term includes several diseases that occur with the deposit of waste proteins that accumulates in perivascular spaces on their way to the lymphatic pathway such as CAA and CADASIL. Failure of elimination results in the accumulation of these proteins, that then formed aggregates. In ageing occurs changes in the extracellular matrix causing the decrease in these process also (43).

The vascular deposit that occurs in AD associated with aging occurs due to a failure in the elimination of these peptides. In the forms of early AD, an increase in production of Aβ is added in these diseases, such as hereditary forms of Alzheimer's disease due to mutations in the genes of presenilin 1 (PSEN1), presenilin 2 (PSEN2) or the amyloid precursor protein (APP) (220).

Perivascular and perilymphatic pathways contribute to Aβ clearance in more than 55-65% of Aβ clearance systems **[Figure 10].** It is a very important pathway of elimination of Aβ (221). Soluble forms of Aβ contained in the interstitial space of the extracellular spaces of gray matter enter the basement membranes of capillaries and drain along PVSs towards subarachnoid space (222). ISF solutes diffused and enter perivascular drainage pathways along the basement membrane of capillary and arterial walls and then move towards the leptomeningeal arteries and ultimately to cervical lymph nodes (23,221,223).

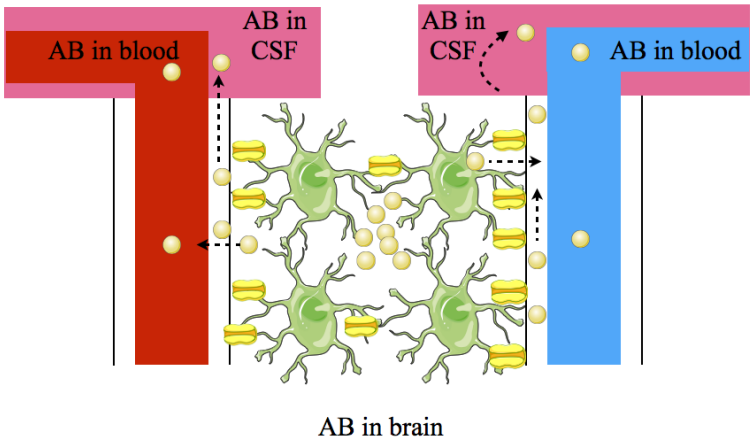


Figure 10: Perivascular and perilymphatic pathways for drainage of brain Aβ. Own figure, modified from (184)

We can distinguish several processes that lead to increase the drainage of Aβ along perivascular pathways **[Figure 11]**. One is the decrease of degradation clearance intracellularly or extracellularly. Soluble Aβ can be removed from the brain by various clearance systems, including enzymatic degradation and cellular uptake. This process is carried out by proteases expressed and secreted mainly by astrocytes and microglia; the peptides are phagocytosed from the extracellular space and degraded intracellularly and extracellularly by different enzymes including Neprilysin, Insulin-degrading enzyme, Tissue plasminogen and MMPs (224–226).

Other system is the absorption of Aβ into the blood. If this process is reduced it results in an increase of drainage of perivascular pathways. The elimination of Aβ from the interstitial space through the BBB occurs through specialized transport systems like LDL receptor family

members such as LRP1, which is the main transporter for Aβ efflux at the BBB, (227–229), and ABCB1, an ATP –binding cassette transporter for Aβ efflux at the BBB which exports directly Aβ into circulation (230,231).

The most important genetic risk identified for late onset AD is the APOE 4 allele (232). APOE4 interacts much less effectively with LRP1 than other APOE glycoproteins, producing a much lower elimination of Aβ from the brain and, hence, promotes its retention (233).

APO E is a glycoprotein produced by the liver with functions in lipid metabolism but also produced by astrocytes in the brain. Humans have 3 alleles of this protein: APOE2, APOE3, APOE4. APOE4 is the most important risk factor for late-onset sporadic AD (234). The presence of an APOE4 allele increases the risk of suffering AD up to 4 times; in the presence of the two alleles this risk goes up to 12 times, compared to APOE3 carriers (235).

Carriers of APOE4 have greater damage of BBB due to pericyte degradation and the activation of the MMP-9 pathway that enhance the degradation of TJ and membrane basement proteins (236).

Phosphatidylinositol binding clathrin assembly protein (PICALM) is involved in the endocytosis of LRP1- Aβ, influencing the Aβ clearance across the BBB (237,238). In AD, endothelium PICALM levels are reduced, accelerating the Aβ accumulation.

Clusterin or Apolipoprotein J (APOJ) is a glycoprotein that binds Aβ, specifically Aβ42 and prevents aggregation and promotes clearance trough the BBB (239).

ABCB1 contributes to efflux the AB into the blood; this protein is down-regulated with normal aging and also by Aβ1-42 itself that exacerbates the accumulation of Aβ.

RAGE is the main the Aβ influx transporter from the blood to the brain. Aβ can entry the brain by RAGE which transports free Aβ from the circulation into the interstitium (240).

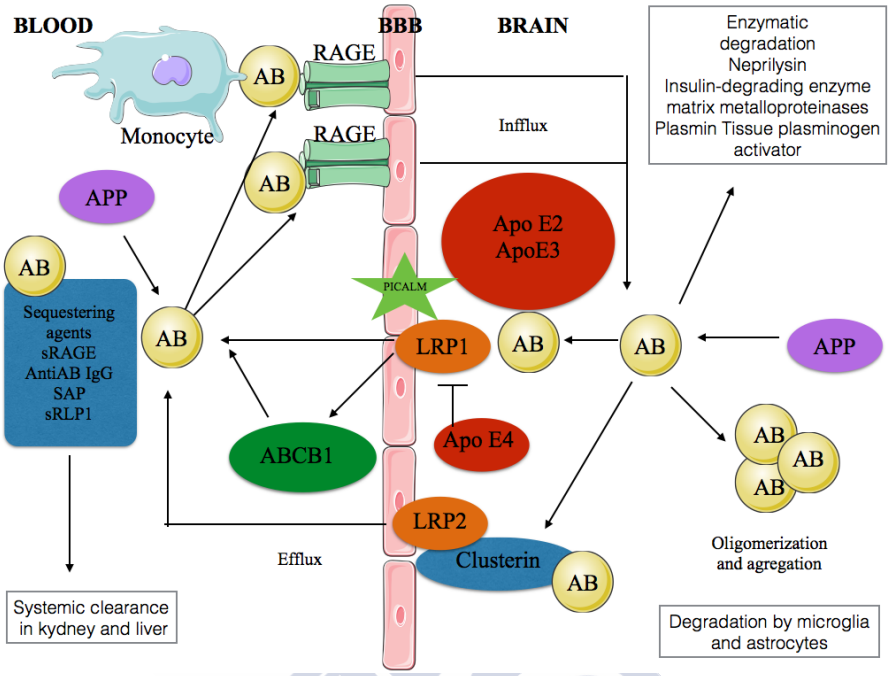


Figure 11: Aβ efflux and influx through the BBB. Aβ can enter the brain via RAGE as free plasma-derived peptide, or can be transported by monocytes. Sequestering agents (soluble transporters to systemic degradation) can prevent the Aβ entry from the circulation into the brain. Aβ is eliminated from the brain enzymatically or by transportation through the BBB. LRP1 mediates the efflux of unbound Aβ and Aβ bound to ApoE2, ApoE3 or alpha 2M from the brain parenchyma into the blood with the help of ABCB1; ApoE4 inhibits this transport process. Aβ bound to clusterin is transported through the BBB by LRP2 (241)**. Own figure (modified from** (241)**)**

RAGE is normally expressed at low levels at the BBB, but its expression is increased in normal aging in AD brain endothelium and is associated with increased cerebrovascular and brain accumulation (242–244).

Also there are evidences in relation to the Aβ accumulation induced by hypertension in animal models of hypertension (245).

Soluble-RAGE was analyzed in animal models of AD for its therapeutic function because it decrease Aβ by interacting with RAGE (246).

Dysfunction of these clearance systems occurs in AD and amyloid angiopathy. Factors that impair Aβ clearance in AD are 1) the expression of blood transporters LRP1 and ABCB1 is decreased and influx transporter RAGE is upregulated, (233,247) 2) in carriers of APOE4 allele, less antioxidant activity occurs than in other isoforms, which mediates a proinflammatory pathway that leads to the dysfunction of the BBB and induces conformational changes in Aβ due to a decrease in pH, causing its elimination (248). Moreover, according to studies on this subject the acid pH would also cause the hyperphosphorylation of tau (249).

There is another clearance system where the Aβ in the CSF can be absorbed by the arachnoid villi or through the perivascular and perineural spaces into the lymphatic system (250,251).

It is important to refer the disruption of perivascular drainage with age and arteriosclerosis, with loss of elasticity and changes in basement membranes in the walls of cerebral arteries.

An adequate arterial pulse wave is necessary because the direction is a movement force reverse (because it is in the opposite direction to blood flow) of the ISF through the PVS towards out of the brain (252). The reduction of elasticity with age reduces the arterial pulse amplitude, leads to stasis of the flow in the PVS and facilitates the deposition of Aβ, conducting to an enlargement of PVS that often become visible on MRI sequences (253).

The movement of the flow occurs thanks to the conformational change of the basement membrane during the systole and the diastole of the cardiac cycle in form of valve-like action (42). The vessels with age become stiffer and less elastic and the changes produced by atherosclerosis also contribute to the loss of arterial contractions necessary for perivascular drainage (254). Biopsies of patients with CAA present arteries with loss of smooth muscle cells, narrowing of the vacuum lumen, thickening of the wall and formation of microaneurysms (255–257).

CLEARANCE SYSTEM	AB
Blood-brain barrier clearance	Majority of A clearance LRP1 efflux ABCB1 efflux APOE-mediated efflux 2M mediated efflux LRP2-mediated efflux RAGE influx
Degradation clearance • Intracellular • Extracellular	Ubiquitin-proteasome pathway Autophagy-lysosome pathway Endosome-lysosome pathway Proteases Glial phagocytosis
ISF flow clearance • CSF sink • Perivascular drainage • Perivascular glymphatic	 Contributes to A clearance Contribution % to A clearance unknown Contribution to A clearance (55-65%) Likely to facilitate blood-brain clearance
CSF absorption clearance • Circulatory • Lymphatic	 Arachnoid villi Blood-CSF barrier transporters (e. g . LRP1 efflux). CSF lymphatic absorption

Table 4: Clearance systems of Aβ. Modified from (241)

7.3.2 The BBB and neurovascular unit in AD

BBB undergoes changes in AD. Dysregulation and disruption of NVU and the BBB may contribute to the onset and progression of dementia and AD (258).

In brain biopsy studies in patients with AD , alterations in the microcirculation are frequently observed, besides the accumulation of Aβ in capillaries there is also a reduction in the density of the

capillaries, decrease in the number of mitochondria, accumulation of collagen IV (259) in the basement membrane, loss of TJs and of AJs , occludin, claudin-5 and ZO-1 (119,260,261), increase fibrinogen leakages in the brain parenchyma, rupture of the BBB and transfer of molecules to the parenchyma from the circulation (262).

There is also loss of pericytes in AD brains that could accelerate brain Aβ deposition and CAA formation (263).

Alterations in the characteristics of astrocytes have also been observed in animal models and in human brains with AD. There is a reduction of the expression of AQP4 that mediates the CSF flux and clearance of solutes in the paravascular space and the drainage of the ISF leading to an impairment of clearance of AB (264,265).

Numerous studies suggest Aβ is toxic to the NVU and causes endothelial dysfunction (266–271). Aβ is likely to disrupt the organization of TJs and AJs in endothelial cells, activating MMPs (119,272). It has even been specifically studied that Aβ vasoconstricts the cerebral arteries by altering the neurovascular junction in neuroimaging studies that use techniques to study functional hyperemia. Functional hyperemia was shown to decrease in exposure to Aβ in these patients (273). Alterations in the metabolic function of cerebral vasculature also occur in individuals with early stages of AD in which the expression of GLUT-1 decreases leading to reductions in glucose uptake in cerebral PET studies. (274–276). It has also been observed that GLUT-1 deficiency initiates early BBB disruption by the reduction in TJ proteins and accumulation of fibrinogen and IgG in mice (277).

An increase in oxidative stress occurs through Aβ that activates the innate immunity through the cluster of differentiation 36 (CD36) receptor of perivascular macrophages and activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, causing cerebrovascular oxidative stress in animal models in which intravenous Aβ was injected. This could be a way to start vascular dysfunction observed in AD (278).

As previously mentioned, the expression of RAGE in the endothelium mediates the transport of Aβ towards the parenchyma and even the high concentration of the peptide mediates the expression of

more expression in the endothelium causing the propagation and amplification of its toxicity (172,244,279).

Dysregulation of the neurovascular unit (e.g., diminished endothelial transport, loss of TJS integrity, basement membrane disorganization, pericyte degeneration, and astrocyte depolarization) is induced during AD progression, which is particularly associated with the Aβ accumulation. These alterations, in turn, contribute directly or indirectly to the disturbed Aβ clearance in the NVU and across the BBB, thus setting up a vicious circle in AD pathogenesis. In parallel, plasma protein leakage, reduced brain glucose uptake, and neuroinflammation caused by BBB damage may lead to further cellular toxicity, making neurons more susceptible to AD pathologies (280).

7.3.3 Neurovascular dysfunction, inflammation and endothelial activation in AD and VaD.

Endothelium as we have seen previously synthesize a significant amount of soluble substances, similar to paracrine and endocrine organ.

Soluble factors include anti and pro-coagulation factors, inflammatory chemokines and cytokines, proteases, growth factors, vaso-relaxation and vasoconstriction factors with effects on nearby cells (281). When endothelial cells suffered injuries products derived of a dysfunctional endothelium could result in neuronal injury in neurodegenerative disease states (282–284). There is evidence from studies where co-culture AD microvessels and neurons leads to neurotoxicity in contrast of co-culture microvessels from younger or elderly non-demented controls (285).

In the plasma of older patients with late onset AD and VaD have been determined elevated levels of markers of endothelial dysfunction (E-selectin, VCAM-1) (286).

Specially VCAM1 has been linked to microvascular damage (damage to cerebral white matter) in patients with AD (287).

Additionally, markers of chronic inflammation, such as elevated plasma concentrations of C reactive protein (CRP), a plasma acute-phase protein and plasmatic cytokines such as IL-6 (288) TNF-,α IL-

1β and IL-10 are increased in patients with AD and VaD suggesting endothelial dysfunction in both subtypes of dementia and low grade of systemic inflammation in these pathologies (289–291).

However, at present, these markers have not demonstrated the ability to discriminate between different types of dementia (292).

There is also controversy in relation with levels of CRP (293) over whether they are elevated or reduced levels in patients with AD, but recently it has been showed lower level of CRP in patients with mild and moderate AD than in healthy controls in a meta-analysis (294).

Injured endothelium releases thrombin and prothrombin potent microglial activator leading to 1) activation of inflammation pathways including monocyte chemoattractant protein (MCP-1), ICAM-1 (295) and angiogenesis (296,297) with overexpression of VEGF, MMPs, (298) IL-1 β, IL-8 and integrins 2) activation of oxidative stress (299) through NADPH oxidase (300) and 3) induction of pro-apoptotic proteins (301).

Circulating endothelial progenitor cells (EPCs) are markers of endothelial dysfunction and can be detected in peripheral blood (302)(303). These cells express CD33+, CD 34+ and the vascular and the vascular endothelial growth factor receptor (VEGF-R) markers that come from the bone marrow and are released into the systemic circulation to repair the damaged endothelium through neovascularization processes (304,305) among others such as reendothelialization and neointima formation (306).

The process of EPCs mobilization is mediated by MMP-9 (307), VEGF or inflammatory cytokine, through stimuli like ischemia or vascular trauma (308–310).

There is ample evidence of the role played by these cells in processes, observing lower levels of EPCs in multivessel pathologies such as atherosclerosis, coronary heart disease and ischemic stroke (311–314) but they have also been studied in neurodegenerative diseases such as AD where decreased CD 34+ EPCs is also found (315).

There are some hypothesis about the reduced levels of EPCs in AD but mainly it is postulated that there is a consumptive loss of EPC activity due to damage of the cerebral microvessels and the exhibition

to the Aβ-rich cerebral environment (316). This condition leads to an impairment in the capacity to repair microvessels (317).

The circulating soluble TNF-related weak inducer of apoptosis (sTWEAK) is a molecule of TNF superfamily, described in 1997. It is considered a cytokine with important functions in immune system as the induction of other inflammatory cytokines. TWEAK was described originally as an inducer of cell death because the molecule is similar to other TNF ligands but TWEAK triggers cell death in a "weaker way", its receptor has not a death domain , and in the presence of TWEAK the cells require long period of incubation and high ligand concentrations to die (318).

TWEAK is a cell surface transmembrane protein but can be secreted to the extracellular space; its receptor is known as TWEAK R or fibroblast growth factor-inducible 14 (Fn14), which contains TNF receptor associated factor (TRAF) binding sequence and activates the nuclear factor kappa light chain enhancer of activated B cells (NFκB) 1 and NFκB2, with multiple cellular responses depending on the cell type and context. TWEAK for example is implicated in functions of stimulation of cell growth and angiogenesis, with important roles in cancer due to the functions in proliferation and cell death **[Figure 12]** (319).

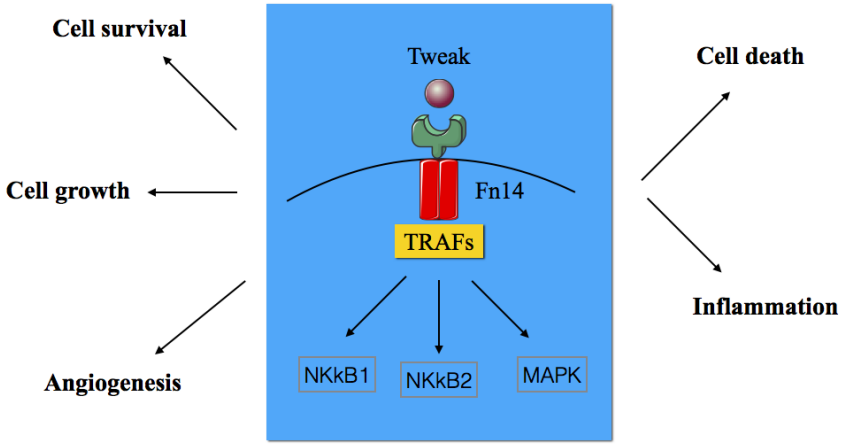


Figure 12: schematic representation of structure of TWEAK and the main functions. Own figure, modified from (320)

TWEAK in acute injury has a physiological role coordinating inflammatory and progenitor cell responses to regenerate tissue, but chronic inflammatory disease is often upregulated with persistent activation and promoting inflammation and tissue damage, with pathological hyperplasia, angiogenesis and gliosis (320).

The TWEAK receptor Fn14 is expressed by epithelial, mesenchymal, endothelial cells, neurons, astrocytes and Schwann cells of the nervous system. Fn14 is inducible by growth factors as fibroblast growth factor (FGF), platelet derived growth factor (PDGF) and VEGF in damaged tissues and in many human tumors.

In humans, circulating TWEAK levels are increased in patients with chronic inflammatory diseases compared with healthy controls.

TWEAK induces chemokines, cytokines, and MMPs and stimulates proinflammatory activity in macrophages to produce Il-6 and Il-8.

The role of TWEAK/Fn14 in central nervous system inflammation has been investigated recently. TWEAK stimulates astrocytes, microglial cells and vascular endothelial cells and induces neuronal cell death. TWEAK promotes astrocyte proliferation and inflammatory activity, including production of Il-6, IL8 and upregulation of ICAM-1 resulting in pathological responses with reactive gliosis. TWEAK is implicated also in increasing BBB permeability and NVU disruption, most likely via astrocyte expression of MMP-9 (321).

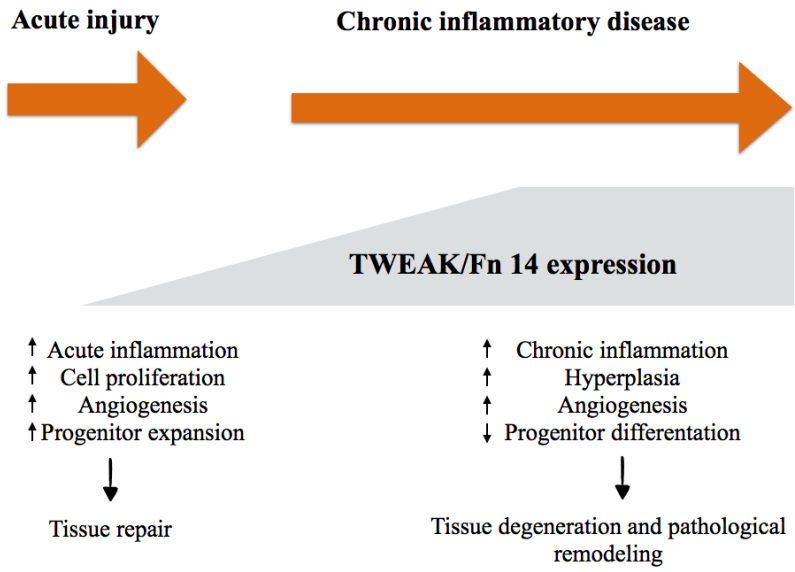


Figure 13: Progressive increase of TWEAK during acute injury and chronic inflammatory disease. Own figure, modified from (320)

7.3.4 Neurovascular dysfunction and neurodegeneration in dementia and Alzheimer´s disease

Vascular insults can initiate a cascade of molecular events leading to neurodegeneration, cognitive impairment, and dementia. There is a link between neurovascular dysfunction and neurodegeneration including the effects of AD genetic risk factors on cerebrovascular functions and clearance of Aβ.

Vascular risk factors, such as hypertension and diabetes, and/or genetic factors for AD, such as APOE4, can lead to cerebrovascular damage (hit 1).

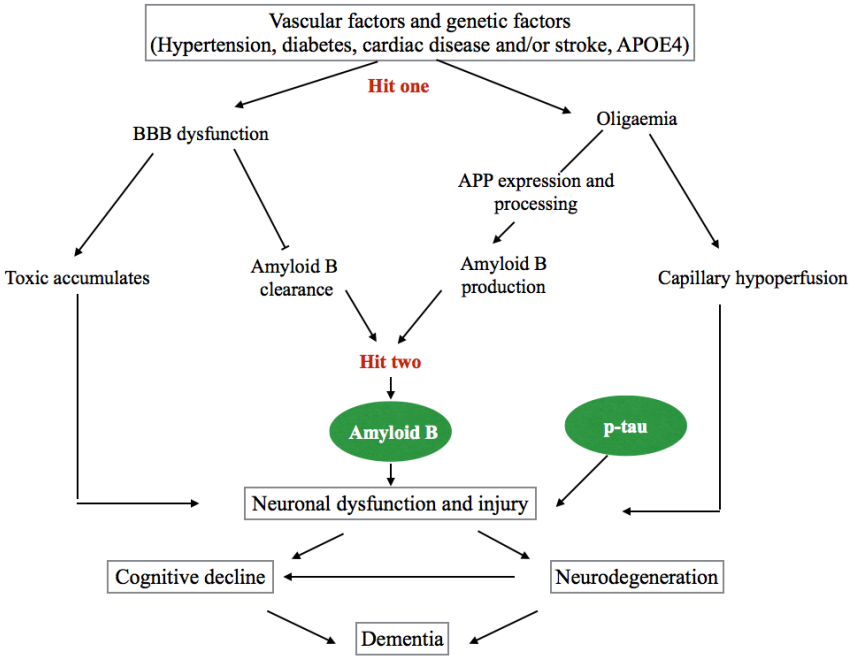


Figure 14: The two hit vascular model of Alzheimer´s disease (AD) dementia. Own figure, modified from (233)**.**

Within the Aβ increase leads to cerebrovascular damage and BBB dysfunction and accumulation of blood-derived neurotoxic molecules in the brain. BBB dysfunction can disrupt Aβ clearance across the BBB and oligoemia leads to overexpression and enhanced processing of APP, which both can promote the Aβ accumulation (hit 2).

These mechanisms lead to synaptic and neuronal dysfunction, neurodegeneration and ultimately leads to dementia (322).

7.4 CEREBRAL AMYLOID ANGIOPATHY: CLINICAL FEATURES

7.4.1 Haemorrhage, superficial siderosis and microbleeds:

ICH is about 15% of acute strokes and has a incidence of 10-30 cases per 100.000 (323). CAA is an important risk factor for spontaneous non-traumatic ICH in the elderly localized mainly in lobar regions (324).

CAA findings in autopsies studies clearly increases with age (325), with percentages of 20% in people between 60-70 years and almost 70% in people over 90 years. CAA is present in almost 99% of Alzheimer disease patients.

The rupture of a small arteries in the leptomeninges and cerebral cortex caused by alterations in the arterial wall is produced by deposition of Aβ in the media and adventitia; amyloid is located there in the path of drainage trough PVS.

The most frequent form is sporadic CAA-ICH but with the increase of use of antithrombotic therapies, there is a higher risk of iatrogenic CAA associated- ICH. There are also familial forms of CAA with hemorrhages (326).

CAA related with ICH can be developed in any region of the cortex, and neurological symptoms and signs differ according to the hematoma location. Clinically, patients with CAA-related ICH present with a focal neurological deficit, a mass lesion and possibly with symptoms of high increased intracranial pressure or with transient neurological symptoms or seizures. The recurrence in the same location is frequent.

Although the exact cause is unknown, the CAA related microhemorrhage has predilection for posterior areas, such as temporal, parietal and occipital lobes (327).

There are some factors that influence the development of hemorrhages such as the presence of hypertension, APOE-epsilon 2 genotype (328) and specific types of MMPs activated by Aβ (mainly MMP-2 and MMP-9) causing breakdown of BBB (329,330).

CAA can present subacute with progressive dementia over the course of weeks to months. Other form, important to differentiate, is Aβ-related angiitis and 20% present ICH; other symptoms are headaches, dementia, seizures, and focal neurological deficits. The treatment consist of steroids and cyclophosphamide (331).

In AD the deposition of Aβ is mainly located in the parenchyma, but in patients with the presence of the APOE-epsilon 2/epsilon4 accelerate formation and deposition of Aβ fibrils in the blood vessel wall and the possession of both alleles is associated with early-onset CAA with recurrent lobar hemorrhages (332).

Superficial siderosis is the haemosiderin deposition in the subpial layers of subarachnoid space of the brain and spinal cord as a result of repeated bleeding caused by rupture of meningo-cortical arteries and capillaries affected by amyloid deposition. It is very often present in patients with CAA and a potent diagnostic marker of CAA (333) (334) even to predict early recurrent lobar ICH (335). But superficial siderosis is typically associated with a syndrome consisting of progressive sensorineural hearing loss, cerebellar ataxia and pyramidal signs (336).

MH or cerebral microbleeds (CMB) are small intracerebral haemorrhages of less than 10 mm in diameter, identified by brain MRI. Histologically are blood extravasations into perivascular space or Virchow-Robin space in hemosiderin-laden macrophages and pericytes without disruption of the surrounding space (337). In CAA, pathologic lobar localization of MH is more frequent than deep hemispheric or infratentorial topography; this localization is more typical of hypertensive microangiopathy (338).

They are considered subclinical but presence of MH is linked to cognitive decline (339–341).

7.4.2 Cerebral infarction and white matter lesions

Cortical microinfarcts and WML are observed in patients with CAA, and they are associated to cognitive decline (342). The mechanism is not completely clear but the main hypothesis is that the deposition of Aβ in the blood vessel walls induces hypoperfusion and reduces vascular reactivity leading to focal ischemia (343).

7.4.3 Dementia and cognitive impairment

According to studies carried out with autopsies of brains with dementia, as the population-based MRC Cognitive Function and Aging Study CAA (344) and Honolulu-Asia Aging Study (345), suggest that severe CAA may be an independent risk factor for cognitive impairment in old age, but in part it may be due to the strong association between amyloid angiopathy and other pathologies such as neuritic plaques (346). In addition CAA causes vascular lesions that contribute to dementia such as hemorrhages or MH and cerebral ischemia.

Advanced CAA causes significant vascular dysfunction representing a subtype of vascular dementia. It is thought that the presence of CAA and SVD in patients with AD contributes to an impairment in cognitive function. Vascular brain injury occurs synergistically with concomitant AD pathology (347).

7.4.4. Clinical imaging expression

CAA is characteristically associated with MRI biomarkers of small vessel brain injury, including strictly lobar cerebral microbleeds, cortical superficial siderosis, centrum semiovale perivascular spaces, and WML. Although these neuroimaging markers reflect distinct pathophysiological aspects in CAA, no studies to date have combined these structural imaging features to gauge total brain small vessel disease burden in CAA (348).

8. SPORADIC NON-AMYLOID MICROANGIOPATHY

The terminology of sporadic non-amyloid microangiopathy is less specific and more difficult to define. It has received several terms such as hypertensive arteriopathy or vascular risk factor related SVD to describe the alterations in the arterial wall of degenerative type usually related to old age, hypertension, smoking and diabetes mellitus and other common vascular risk factors. Another term for which it is known is arteriolosclerosis (1).

This type of microangiopathy is actually a systemic disease, since it is observed in other very perfused organs like retinas and kidneys. The etiology of this systemic disease is attributed to the endothelial dysfunction produced by the effect of aging on the arteries exacerbated by vascular risk factors (349).

8.1 NEUROPATHOLOGY

Histopathologic alterations observed in this entity are degenerative changes in the vessel wall which are part of the concept of atherosclerosis. Atherosclerosis is the hardening of an artery and it is the vascular disease that causes higher mortality in the industrialized countries (350). Atherosclerosis is an inflammatory disease; the mechanism that initiates the process of endothelial dysfunction and promotes the inflammation will be reviewed later (351).

There are three lesions in the category of arteriosclerosis: atherosclerosis, arterioloesclerosis and Mönckeberg medial calcific sclerosis (352).

Arterioloesclerosis is the lesion that affects the arterioles and small arteries that have 1 or 2 layers of smooth muscle. It is observed manly in the deep arterial perforators at the base of the brain supplying the basal Ganglia, thalami and brainstem structures (15,353,354).

There are two subtypes of arterioloesclerosis, the hyaline type and the hyperplastic type. Other pathological features include fibrinoid

necrosis, distal manifestations of atherosclerosis (microatheroma) and microaneurysms (4). These pathological findings have been described in brains of hypertensive patients with lesions of lacunar infarcts and is often found in arterioles adjacent to deep brain ICHs (353,354).

Hyperplastic type includes fibromuscular intimal thickening habitually seen in transplant vasculopathy, restenosis lesions after balloon angioplasty or stenting, and nonspecific intimal thickening as occurs in temporal arteries with ageing (355). This subtype due to its specific etiology is not of interest in this field.

Hyaline means translucent or transparent homogeneous substance "like glass", and is characterized by thickening of the basement membrane due to the deposit of degenerated collagen synthesized by endothelial cells and smooth muscle as well as amorphous masses of acid mucopolysaccharides (356).

Narrowing of the lumen occurs as well and a loss of smooth muscle cells from the tunica media (357).

Hyalinization is a common finding in arterioles of organs affected by hypertension but it is thought that it is not to be the immediate cause of the rupture of the blood vessels. Fibrinoid necrosis is much more frequent in patients who have suffered brain hemorrhage than in hypertensive brains without hemorrhage and is thought to be a point of weakness of the arteriolar wall where the extravasation of blood occurs (358). Fibrinoid necrosis is produced by the insudation of plasma proteins into the arteriolar wall which have crossed through the injured endothelial cells (359). Fibrinoid necrosis is characterized by the replacement of the vascular wall by granular eosinophilic material, consisting of deposits of fibrinogen (fibrin), immunoglobulins and immunocomplexes, usually accompanied by endothelial swelling by IgG deposits and protrusion of these cells into the vascular lumen; it is the one most specifically associated with hypertension, (360) which is more common in hypertensive patients´ brains than in those without hypertension and is often found in arterioles adjacent to deep parenchymal brain haemorrhage (353).

There was an important advance in ideas about etiology and pathogenesis of SVD and arteriolosclerosis due to brain autopsy studies done by C. Miller Fisher between 1955 and 1973.

Fisher, in a work of ten lacunar infarcts, described atheromatosis of perforating arteries in six of the cases, in the remaining four, he observed lesions of lipohyalinosis in two and attributed the other two to embolisms, since arterioles appeared normal as if the embolism had been dissolved (15). Fisher also observed the presence in an advanced degree of a hypertensive disease process affecting small arteries in the parenchyma surrounding a deep brain hemorrhage such as fibrinoid necrosis or lipohyalinosis.

There is a confusion arising from the substitution of the term lipohyalinosis for fibrinoid due to Fisher use of the term lipohyalinosis to refer to fibrinoid necrosis lesions, because this type of lesions also contained lipids according to the general conviction (361).

Other alterations observed in the small arteries surrounded deep brain hemorrhage were microaneurysms, occlusions and small infarcts (353). Microaneurysms or miliary aneurysms, described by Charcot and Bouchard in 1868, are considered a point of less resistance of the vascular wall (362); their formation is associated with the loss of pericytes and the stability of the microvascular wall (363) Microaneurysms are more frequent in hypertensive patients (364) and usually coexist with necrosis fibrinoid in cases of intracerebral hemorrhage (361,365,366).

These aneurysms also have been reported in other diseases such as amyloid angiopathy (200). Also, spontaneous intracerebral hematomas are frequently associated with ischemic lesions due to stenotic vessels, related to arteriolosclerosis microangiopathy and blood-brain barrier breakdown (367).

8.2. PATHOGENESIS: BLOOD BRAIN BARRIER, NEUROVASCULAR UNIT AND ENDOTHELIAL DYSFUNCTION IN SPORADIC NON-AMYLOID MICROANGIOPATHY

The microvascular damage is linked in this type of microangiopathy to the presence of vascular risk factors, mainly hypertension and diabetes mellitus and aging. Vascular alterations such as fibrosis, arterial stiffness and thickening of vascular lumen are

produced. These vascular changes cause a reduction in cerebral blood flow that produces hypoxia and ischemia.

Through studies, mainly developed in animal models of brain microangiopathy, the following molecular cascade has been observed.

Hypoxia and ischemia trigger a molecular cascade that leads to the induction of HIF1α and oxygen-sensitive genes (38,368).

There is an increase in the expression of erythropoietin and VEGF that stimulates angiogenesis, in addition cytokines such as TNF α, interleukin 1 β (IL1β) and NF-κβ that cause inflammation are activated (369–371). HIF-1α /hypoxia increases a convertase called furin that actives MMPs (93,372) **[Figure 15].**

The pathological molecular cascade includes the production of MMPs, specially MMP-2, that plays an important role in the pathogenesis of breakdown of BBB and inflammation in conditions of cerebral hypoperfusion (373); other implicated are MMP-3 and MMP-9 (99). Free radicals like reactive oxidative species (ROS) and other enzyme enzymes such as Cicloxigenase-2 (COX-2) are also produced (374). This cascade ends with the breakdown of the BBB with the production of vasogenic edema and myelin rupture and death of oligodendrocytes, causing demyelination (375) **[Figure 16].**

Oligodendrocytes are very sensitive to hypoxia and ischemia, producing degenerative changes in them in patients with chronic hypoxia (376). In patients with WML, a smaller number of oligodendrocytes and a lower remyelination capacity have been documented (377).

The demyelination that occurs is not through autoimmune processes as it happens in other pathologies and it has been called by-stander demyelination (39).

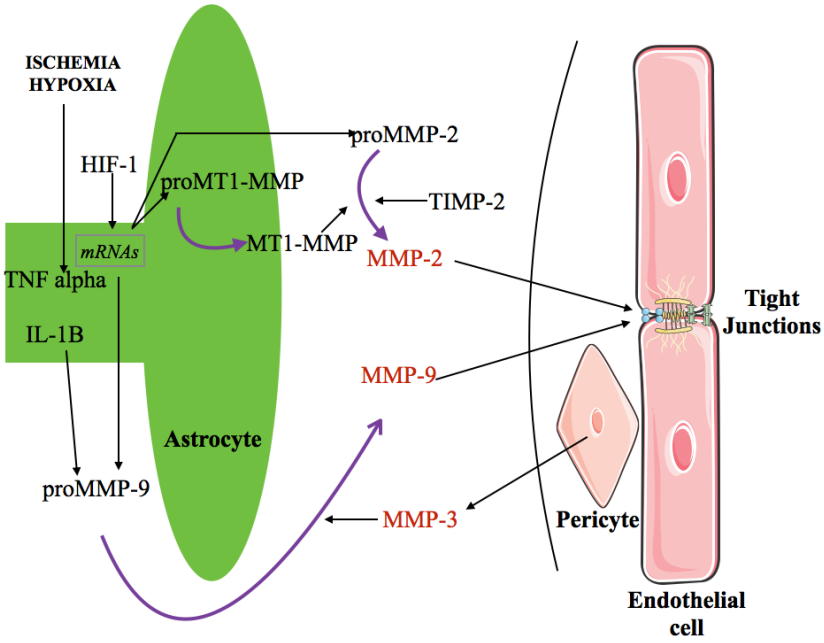


Figure 15: schematic drawing of the processes of activation of MMPs via astrocyte end feet induced by ischemia and hypoxia. The activation of MMP-2 occurs through the action of the proMMP2 and TIMP2 , the activated MMP2 leading to breakdown of BBB. When the hypoxia is prolonged and severe there is an expression of MMP-9 and MMP-3 leading to further tissue destruction. Own figure, modified from (99).

The association between BBB rupture and dysfunction and white matter damage has been well documented over the past few years (378). Studies were performed that showed an increase in the permeability of BBB with contrast-enhanced brain MRI (images processed in unusual sequences such as multiple fast T1) (379) and also through studies that observed an increase in albumin in the CSF in patients with cognitive deterioration of vascular cause and WML (380).

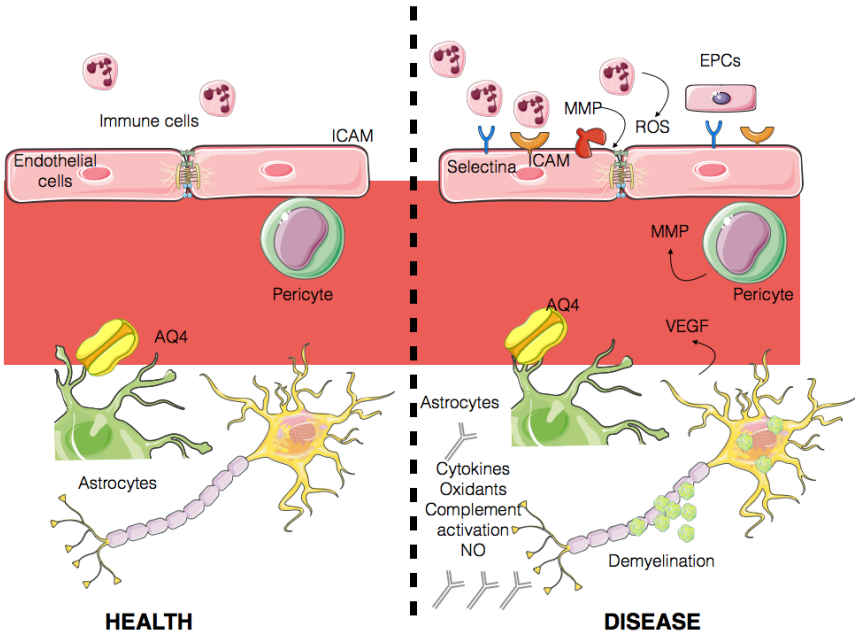


Figure 16: schematic drawing of the pathogenesis of sporadic SVD. Structural alterations in endothelial cells caused by MMPs, ROS, and immune cells with the breakdown of the BBB produce vasogenic edema and myelin rupture and death of oligodendrocytes, causing demyelination and neuroinflammation. Own figure, modified from (81)

The inflammatory response attracts microglia and macrophages that are activated and produce proteases that destroy the basal lamina and TJs in the ECs and attack the myelin fibers (40,375,381).

In patients with white matter damage it has been observed that they express markers of apoptosis and that they decrease in number. In turn, an increase in astrocytic clasmatodendrosis occurs, which involves cellular changes characterized by cytoplasmic edema, fragmentation of the dendritic processes and processes of cellular autophagy (382).

Activated astrocytes contribute to damage in the interstitial matrix in an attempt to perform a repair process because they produce proteins

that are deposited in the matrix and chondroitin sulfate proteoglycans that contribute to vascular fibrosis (77,383).

Other changes observed in astrocytes in this context is the displacement of AQP4, a molecule that functions as a water channel and which is responsible for maintaining the balance between fluids and preventing the development of edema (127).

Alterations have also been observed in the relationship between pericytes and ECs. ECs recruit pericytes via PDGF β and PDFG β receptor; in patients with SVD a reduction of this signal has been observed leading to a lower number of pericytes in the vascular wall that causes capillary rupture, tortuosity and formation of microaneurysms and microbleeds (384).

8.3 SPORADIC NON-AMYLOID MICROANGIOPATHY: RADIOLOGICAL PHENOTYPES:

8.3.1 White matter hyperintensities or white matter lesions:

WMH or WML are a radiological phenotype and a part of the spectrum of SVD which includes lacunar (or small subcortical) ischemic and hemorrhagic stroke, lacunes, microbleeds or MHs, perivascular spaces and brain atrophy.

The term leukoaraiosis (LA) was used for the first time in the late 1980s by Hachinski to refer to low attenuation or hypodensity located in periventricular and white matter area in brain CT. These structures are typically affected by WMH because are border zones of vascular territories (27).

Brain MRI studies are more sensitive to detect these areas, are hyperintense in T2 and FLAIR and represent a subtle alteration in water content. Earliest MRI studies with 0,2 or 0,5 Tesla were less sensitive than nowadays MRI studies with 1,5 or 3 Tesla.

In relation to the nature of the WMH could be the result of demyelination, axonal loss and ischemic process. Progressively periventricular and deep WMH can increase in size, occasionally shrink and lead to atrophy of white matter and overlying cortex. Also acute

small subcortical infarct may develop a WMH cap and project its effects onto overlying cortex and brainstem causing thinning (385).

The severity of WMH is measured by Fazekas scale, a visual scale which evaluates the degree and confluence of WMH.

The absence of WMH is considered normal. Grade I is the hyperintense periventricular capping of the frontal horns. A smooth halo of periventricular hyperintensity has been linked to disruption of the ependymal lining, subependymal zone gliosis and concomitant loss of myelin. Punctate lesions in the deep and subcortical white matter corresponded to minor perivascular reduction in myelin content possibly because of a lower permeability of thickened arteriolar walls; it could be normal in people older than 65 years.

Larger patchy and confluent hyperintensities, however, appear to indicate more extensive ischemic damage consistent with advanced microangiopathy. The incipient confluence is considered grade 2 and can be normal in people older than 75 years but it is pathological in younger people. An important confluence is never normal and is considered grade 3 (386,387).

WMH are more common with advancing age and are associated with progressive cognitive impairment and increase the risk of dementia and stroke. The prevalence of WMH increases with risk factors, including hypertension, diabetes mellitus, smoking, dyslipidemia (388).

Progression over time of WMH in elderly subjects is analyzed in The Austrian Stroke Prevention Study and they observed an increase of 1,1 cm (3) over an observational period of 4 years in healthy subjects (29,389). Van Dijk et al in 2004 in the Rotterdam study published a prospective study of 1077 healthy elderly patients with a mean follow-up of 4 years and observed that silent brain infarcts and severe grade of WMH is associated with an increased risk of stroke independently and the incidence of silent brain infarcts on MRI in the general elderly population increases with age (30).

Hypertension is the risk factor most associated with the progression of leukoaraiosis. In a study with a follow-up of 4 years of patients between 59 and 71 years, it has been observed that patients with better blood pressure control have a lower risk of presenting severe

LA (31). Mainly diastolic blood pressure predicted lesion progression (29).

Other studies have also indicated that active treatment of blood pressure can stop or delay the progression of WMH after a follow-up of 36 months with a combination of antihypertensive perindopril and indapamide versus placebo in a sample of patients with lesions of white matter in the basal MRI (390).

Some polymorphism of angiotensinogen gene was associated with an increased risk for lesion progression independently of arterial hypertension (391).

After the results observed in the cohort of young patients from the Framingham study it is suggested that cerebral vascular damage starts insidiously during life, with a detrimental effect of arterial hypertension already in young adults (392).

Hypertension could directly affect the brain or through a systemic arterial stiffening process that represents microvessel arteriosclerosis with vascular endothelial dysfunction, where cerebral autoregulation is lost secondarily; this effect is known as Tsunami effect because the hemodynamic stress and pulsatile pressure is transmitted towards the brain parenchyma and lead to cerebral SVD (393).

In relation to the effect of arterial hypotension as a risk factor for cognitive progression in elderly patients due to hypoperfusion, there is a prospective study The SMART-MR study, that found an increased risk of subcortical atrophy in patients with low diastolic blood pressure. During the follow-up, among the limitations of the study, it was highlighted that measurements were made in a single-day in the clinic with possible white coat effect and patients with carotid stenosis were included, some of them with stenosis greater than 70% (394). Recently in the Mayo Clinic Study of Aging was investigated the presence of blood pressure slopes as risk factor for the presence of cerebral microinfarcts and it was observed that subcortical microinfarcts at autopsy are associated with declining blood pressure (395).

Diabetes mellitus in several studies showed controversies in its association with the severity of WML. Cross-sectional studies have suggested no association and a meta-analysis did no find consistent association (396,397).

But in prospective studies like LADIS study, Gouw followed 396 elderly subjects for 3 years and found that diabetes mellitus affected the progression of WMH (398). Also in the Second Manifestations of Arterial Disease-Magnetic Resonance study where patients with diabetes mellitus showed a larger baseline volume and a more accelerated increase of WMH (399).

It has been published recently that diabetes is associated with progression and severity of WMH as well, specially more-severe diabetes with higher HbA1c and longer disease duration (400). Hypoglycemia was not associated with the progression in this study.

Dyslipidemia is a very important risk factor mainly in older hypertensive patients (401). Obesity and metabolic syndrome they have also been related to the increase in probability of presenting WMH (402,403). In particular, visceral obesity has been associated with higher rates of WMH in normal cognitive and mild age subjects in a study that used to determine visceral fat waist circumference measurements (404,405).

Several cross-sectional studies have investigated obesity in relation to changes in white matter integrity using DTI. Results indicate that obesity could accelerated the normal aging process probably trough the low grade inflammatory response produced by adipose tissue causing white matter microstructural changes (406).

However in this respect there are contradictory results, because some studies have found a relation in metabolic syndrome with decelerated cognitive decline in elderly (407).

8.3.2 Cerebral microhemorraghes

MHs as we explained above are small, less than 5-10 mm (408) rounded, homogeneous haemosiderin deposits formed by blood breakdown products located in brain macrophages adjacent to small blood vessels that cause radiological artifact (“ susceptibility artifact “) detected by specific MRI sequences like susceptibility weighting imaging (SWI) and gradient echo (T2 *) as hypointense lesions (36). MH were initially described in 1996 (409).

Microaneurysms or pseudoaneurysms are the origin of MHs as well ICH, they are not chronic in all cases, since erythrocytes have been found (410).

MHs mimics can include blood products or not. In the first case are partial volume artifacts, blood vessels, and paramagnetic substances (e.g. calcium or iron). In the second case are cavernous malformation, traumatic microbleeds, hemorrhagic metastases and hemorrhagic transformation of cerebral infarcts.

Prevalence of MHs in elderly healthy subjects without cerebrovascular disease is about 5%; this prevalence increases in patients with SVD up to 57% and 68% in patients with spontaneous ICH (411). In patients with stroke MHs are detected in 34% (33).

By consensus MHs are divided topographically, in deep or infratentorial (typical of the hypertensive microangiopathy) and strictly lobar (typical of the CAA). In patients with AD, MHs are present in one third of the cases and are located in subcortical areas and occipital lobes mainly. There is an important association with apoE genotype as we have seen previously.

MHs located in the basal ganglia or in infratentorial brain regions are related to hypertensive vasculopathy. But up to 15% of patients with ischemic stroke have MHs in both locations, with a mixed pattern (412).

MHs often coexist with other SVD markers like WMH, microinfarcts, lacunes and PVSs (348,413). Topography of MHs and PVSs are useful to determine the etiology of SVD (412).

Aging and vascular risk factors mainly hypertension (OR 3,9, 95% CI 2,4-6,4), are associated with major risk of MHs. Hypertension is the strongest factor in general population and in stroke patients. Diabetes mellitus also associated with greater risk of presenting MHs (OR 2,2 95% CI 1,2-4,2) (33).

Blood pressure (BP) obtained by ambulatory BP monitoring (ABMP) correlates with hypertensive target organ damage and this study shows how nocturnal hypertension referred to as nocturnal non-dipper patterns increases the risk of MHs in 5 to 6 times (414).

8.3.3 Perivascular spaces

As explained before, the lymphatic pathways of the brain begin in the PVSs that surround the small perforating arteries and that separate the blood vessels from the cerebral parenchyma (415).

Normal PVS s are not seen in brain MRI sequences and can only be visualized when enlarged, known as EPVS (416). Is a frequent finding in patients with SVD and are considered one of the earliest neuroimaging markers (36).

They are spaces of less than 3 mm of diameter, filled with CSF-like fluid. EPVS are found in conjunction with other neuroimaging markers of SVD.

EPVS and WMH share the same location, and WMH appear around EPVS; it is suggested that they are a place of CSF stasis by altered glymphatic function and that they intervene in the pathogenesis of the SVD causing edema in the white matter producing the initial lesions of white matter (182,417).

Occasionally EPVS are very enlarged, known as giant cystic Virchow Robin spaces and mimic a cystic mass lesion but in FLAIR images there are perilesional WMH (418).

Topography of EPVS is different between arteriolosclerosis microangiopathy, where they are located in basal ganglia mainly, and in CAA where EPVS are located juxtacortical and the severity is associated with the degree of PVSs dilation (419).

They are independently associated with SVD risk factors like hypertension and aging (420) and they are biomarkers of vascular risk; their presence increase vascular events like myocardial infarction and stroke (as shown in the Northern Manhattan Study) (421). Also they are associated with mild cognitive impairment in hypertensive patients and with the presence of microalbuminuria reflecting the presence of target organ injury in the kidney (422).

8.4 SPORADIC NON-AMYLOID MICROANGIOPATHY: CLINICAL FEATURES

8.4.1 Dementia and vascular cognitive impairment

Small vessel disease is the most common cause of VCI according to community-based autopsy studies (423).

Currently the term VCI is used more than vascular dementia (VaD); it is a syndrome not a disease, because it has a heterogeneous nature with multiple causes (424,425). Furthermore, VCI refers to all forms of cognitive deficits ranging from mild cognitive impairment to dementia.

The subtypes that are included under this term are multiinfarct dementia due to multiple cortical infarcts; small vessel dementia or subcortical vascular dementia (previously known as Binswanger´s chronic subcortical encephalopathy) (426) caused by microangiopathy with lacunes, WML as main features of neuroimaging and pathologically infarcts, demyelination and gliosis; strategic infarct dementia due to an infarct situated in strategic location like thalamus (427), dementia by watershed infarcts caused by hypoperfusion; hemorrhagic dementia that could be associated with CAA ; hereditary vascular dementia by CADASIL and AD with cardiovascular disease characterized by combination of vascular changes and atrophy and mixture of vascular and degenerative pathology (428).

VCI occurs in two main settings 1) post stroke in which cognitive impairment is immediately subsequent to stroke and 2) without stroke where the onset of cognitive decline is in absence of recent stroke but vascular brain injury manifests on neuroimaging or neuropathology (429).

Diagnostic and Statistical Manual of Mental Disorders (DSM) 5 defines VCI as a major neurocognitive disorder of progressive course with alteration of memory that can be present or not and can affect other cognitive spheres (like executive function, attention, visuospatial abilities, judgment, reasoning and emotional control); these alterations interfere in the activities of the patient´s daily life. Diagnosis must be based on cognitive testing involving a minimum of 4 cognitive

domains, including executive /attention, memory, language, and visuospatial functions.

A diagnosis of VaD requires deficits in at least 2 domains, whereas deficits in a single domain are sufficient to diagnose MCI.

In relation to the diagnostic criteria of VCI , there should be evidence of a vascular contribution to cognitive deterioration from some combination of history, physical examination, cognitive profile, and diagnostic testing including neuroimaging. The onset of cognitive deficits should be temporally related to one or more cerebrovascular events or evidence for prominent decline in complex attention (including processing speed) and frontal-executive function.

The prevalence of VaD varies among studies, but a prevalence of 15% is estimated. The differences are due to the different diagnostic criteria applied and often pathological substrates of other dementia such as Aβ and tau usually co-occur (430).

Vascular risk factors for VaD broadly overlap with those for stroke. There is a strong evidence for ageing, hypertension and diabetes mellitus, and some evidence for female sex, smoking, physical activity, obesity and body mass index, dyslipidemia (431).

Also there is an association with genetic factors, mainly with polymorphisms of APOE, ACT, ACE, MTHFR, PON1 and PSEN-1 genes, but only APOE4 and PSEN-1 genes remained significant (432).

8.4.2 Vascular cognitive impairment neuroimaging features

The most frequent cause of VaD is cerebral microangiopathy that is typically manifested with WMH or LA and small cystic cavities, the lacunes, with a signal identical to CSF. These lesions are very common in patients with VaD and other ageing related dementias (433,434).

But not only subcortical alterations are observed, cortical changes derived from subcortical damage like atrophy via degeneration of connecting fiber tracts and also microscopic vascular lesions are recently considered clinically relevant (435).

There is a correlation, well documented in several studies, between the burden of subcortical ischemic lesion and cognitive impairment

even with a threshold effect for WMH on cognitive impairment (436,437).

Presence of WMH causes a two-fold increase in the risk of cognitive impairment and also increase the risk of depression in late life (438,439).

Silent brain infarcts (SBI), that are infarcts observed in neuroimaging techniques without a history of transient ischemic attack (TIA) or stroke, also increase the risk of dementia in double according to Rotterdam scan study and similar in the Framingham study. Their prevalence is high in subjects over 65 years, more than 10%. Most SBI are lacunes attributable to SVD. Thalamic infarcts are associated to decline in memory and infarcts in other locations to psychomotor decline (440).

Cerebral microinfarcts (CMI) are invisible lesions that measure < 1mm and are detected microscopically during pathological examination; they may be located in cortical or subcortical regions and are attributed to SVD (423). CMI have been associated with higher risk of dementia in autopsied Honolulu-Asia Aging Study participants (441).

MHs and cerebral superficial siderosis (cSS) are frequent in patients with VaD; MHs, specially in lobar location, are associated independently with cognitive impairment and with incident dementia in the Rotterdam Scan Study (442). MHs may affect cognition disrupting structural connectivity and network function (443). cSS represents linear deposits of blood breakdown products within the subarachnoid space. cSS is related to CAA and the presence of an apolipoprotein E e2 allele (444).

EPVS are the spaces surrounding the vasculature and have a role in the clearance of fluid and metabolic waste, located in basal ganglia, centrum semiovale and mesencephalon (445). They are associated with markers of SVD and with cognitive function and dementia, however they are not specific for microangiopathy because they are present in other pathologies such as AD and multiple sclerosis (446). In addition, they are not specific to cognitive impairment either, since they are alterations observed in the context of the presence of others markers of cerebral microangiopathy.

8.4.3 Vascular cognitive impairment cognitive evaluation:

Neuropsychology testing is useful tools for the diagnose and monitoring of dementia. However, its their clinical usefulness for differential diagnoses of AD and VaD has been controversial. One reason is that VCI is not a homogenous condition, but encompasses many heterogeneous clinical syndromes, and can result from a variety of pathogenetic mechanisms. VCI, which has SVD as the main vascular cause, is more homogenous than other VaD, and representative groups of patients show more predictable clinical features, including cognitive slowing, dysexecutive syndrome, gait disturbance and incontinence, and have similar natural histories, outcomes and treatment responses. The clinical course of VCI may be gradual, like AD, and step-wise deterioration and focal neurological signs may be subtle, so clinical differentiation of VCI from AD can be difficult. Cognitive dysfunctions of AD are mostly associated with temporo-parietal dysfunction, and cognitive dysfunctions of VCI are associated with the disruption of frontal-subcortical circuits. These different neuronal circuit dysfunctions of AD and VCI may be reflected in scores from certain domains of the Addenbrooke Cognitive examination (ACE).

There are different test to evaluate the cognitive state, one of the most used is in Spain is the Spanish version of the Addenbrooke Cognitive Exam (Addenbrooke's Cognitive Examination Spanish Version, ACEVE) (447). Developed and validated in English by Mathuranath et al in year 2000 (448), it is a brief instrument that is used to detect cognitive dysfunction in the initial stages of dementia. From this test, the VLOM coefficient (verbal fluency + language / orientation + memory as a deferred recollection) can be obtained, which allows orientation on the type of dementia that the patient could have, differentiating between frontotemporal dementia (FTD) or AD as well differentiate between AD and VCI, based on differences commonly seen in the cognitive profiles of these conditions (449).

The maximum score of the ACE is 100 points, of which 30 belong to the Mini-Mental State Examination (MMSE), which are distributed in 6 cognitive domains: orientation (10), attention (8), memory (35), verbal fluency (14) language (28) and visuospatial skills (5). The

orientation and attention components were taken from the MMSE. In relation to memory, it evaluates both semantic and episodic by means of the three elements of the MMSE, adding an address that allows evaluating learning and deferred memory. By incorporating the study of episodic memory, it makes it easier to detect AD in the initial stages.

On the other hand, the evaluation of the language is carried out through tests of denomination of 12 drawings, understanding of orders, repetition of words and sentences, reading of regular and irregular words and writing of a sentence. In addition to this, it includes the assessment of verbal fluency, both phonological (words that start with the letter P) and semantics (category of animals), which allows detecting frontal alterations (448).

Finally, the visuospatial skills are evaluated through the copy of the MMSE pentagons, including, in addition, the copy of a cube in three dimensions and the drawing of the clock, which allows a better evaluation of the alterations in visuospatial and visuospatial abilities.

It is important to emphasize that the ACE allows to counteract the limitations of the MMSE, since despite being the most used screening instrument in late adulthood, presents problems to detect dementias in early stages. One of the causes of this inconvenience is the simplicity of the amnesic and linguistic tasks that make it difficult to detect minor memory and language problems. On the other hand, the MMSE has little sensitivity for to detect dysexecutive syndrome, characteristic in FTD and VCI. Among the differences that can be observed in both tests, it stands out that in the ACE, unlike the MMSE, serial learning and verbal fluency can be evaluated.

Among the improvements present in the ACE is the expansion of the study of language through an increase in items in the denomination task, a deeper evaluation of both the reading of words and the understanding of orders. In comparison, the MMSE, they add tests of verbal fluency, the evaluation of visuospatial functions is extended, including the drawing of a cube and a clock, which are also useful for detecting symptoms of frontal dysfunction (450).

Another advantage that the ACE has, is that by including the MMSE it is possible to apply it in those subjects who had previously

been evaluated with that test and thus be able to observe its evolution, thus allowing the established baseline to be maintained (450).

In a study by Junco and Prieto (2014), they found that the items of greatest difficulty are those related to the learning curve, deferred memory, verbal fluency and the drawing of clock hands. The easiest items are the MMSE denomination, fixation, understanding of simple orders, the first reading item and the repetition of the word "test". On the other hand, the tasks that showed the greatest efficacy to detect dementia are those corresponding to the recall of the day of the week, the last two items of calculation, the last four items of reverse spelling, the deferred memory of three words and the address, verbal fluency, denomination, the drawing of the clock and that of the cube.

The Spanish translation and adaptation of the ACE modifies one of the tasks that evaluates learning and deferred recollection, changing the direction that the patient has to memorize, but retaining the original extension. In addition, the tasks that evaluate language were adapted and the public figures were replaced by their Spanish correspondents (447).

8.4.4 Lacunar stroke

Cerebral infarction of the lacunar type is known as lacune, that means cavity or small size hole.

Lacunar infarcts is a defined subgroup of Oxford-shire Community Stroke Project (OCSP) as LACI, produced by infarct of the territory of the deep perforating arteries. Later the TOAST in 1993 developed a new system and these infarcts are named small-vessel occlusion, and are defined by lesion < 15 mm in diameter on imaging and limited to cases with one traditional lacunar syndrome and no evidence of cerebral cortical dysfunction.

LI account for one quarter of cerebral infarctions and generally develop in patients with hypertension and/or diabetes mellitus.

Fisher in the 1950s, described the classical or typical lacunar syndromes (14,15,451).

- Pure motor hemiparesis: it is the most frequent syndrome, caused by the involvement of motor fibers that are located together, for example in anatomical areas of the basal ganglia in anterior limb of internal capsule, corona radiata, centrum semiovale or pons, which receive their vascular supply by deep perforating arteries or basilar artery branch.
- Pure sensory syndrome: are the smallest of the symptomatic deep infarcts, often caused by infarction of thalamus or the anterior thalamic radiation.
- Sensorimotor syndrome: with a prevalent infarction in the thalamo -internal capsule-corona radiate region adjacent to putamen.
- Ataxic hemiparesis: caused by lacunas in the posterior limb of internal capsule.
- Dysarthria clumsy hand syndrome: patients in this case have pontine infarctions contralateral to the symptomatic side involving corticospinal and cerebellum-thalamo-cortico-pontine-cerebellum tracts.

Most frequent atypical lacunar syndrome are dysarthria with central facial paresis, isolated dysarthria, isolated hemiataxia and hemichorea-hemiballismus.

SVD by atheromatous branch disease and lipohyalinosis, are the most frequent causes of lacunar stroke; other less frequent causes are cardiac emboli, embolism from artery-to-artery atheroma or intracranial stenosis. Embolic stroke are more frequent located in basal ganglia than in centrum semiovale, where they are more likely caused by SVD (452).

Risk factors as well as the secondary prevention therapy was widely studied in the Secondary Prevention of Small Subcortical Strokes (SPS 3) trial and in other studies. Main risk factors are age, male sex, hypertension, diabetes mellitus, heart disease, carotid atherosclerosis and smoking (453).

Age is a non-modifiable risk factor for the development of lacunar infarcts and leukoaraiosis (454,455)

Hypertension is a major independent risk for stroke in general, and is strongly associated with the risk of presenting LI specifically, more than other types, such as large-artery atherosclerosis (456).

Diabetes mellitus is also an independent risk factor for lacunar stroke and have a particular profile with more intracranial atherosclerosis, with preference for posterior territory location and with more risk to recurrent stroke and worse recovery (457).

Smoking as an isolated risk factor increases the risk of presenting a stroke of any etiology, including LI (458).

The Osaka Study for Carotid Atherosclerosis showed that carotid stenosis had an association with the development of LI during the follow up (459).

Cardiac embolism (for example due to atrial fibrillation) is the only demonstrable etiology found in 4% of LI and its role in the etiology of LI is infrequent (460).

LI can be presented clinically as asymptomatic LI in form of silent lacunes and they are due to lipohyalinosis, as transient cerebral ischemia and as a lacunar clinical syndrome (461).

Location of the infarct and its size determine the symptomatology of the patient; lesions near or located in descending tracts of areas of motor or sensory areas produce symptoms, but those that are clinically silent are located in other areas such as the semiovale center, capsule external, basal ganglia and brainstem (462).

For the diagnosis apart from anamnesis, physical and neurological exploration, brain MRI is the technique of choice with high specificity and sensitivity; CT sometimes is not able to detect small lesions (< 2 mm) and, in addition, it may not identify lesions in the brainstem or identify silent lesions (463).

Transcranial Doppler is useful to determine if LI is produced by an intracranial stenosis of the middle cerebral artery or basilar artery that affects the origin of the lenticulostriate arteries or paramedian branches correspond to the stenosis segment.

Moreover, PI and RI can be elevated, reflecting increased vascular resistance. In the past decades it has been described that impaired cerebrovascular reactivity is associated with an increase of the risk of LI (464,465).

8.4.5 Intracerebral haemorrhage

Primary intracerebral hemorrhage (ICH), referred to spontaneous rupture of a cerebral blood vessel without underlying injury, is the most frequent cause of ICH. It is the second cause of stroke, after ischemic stroke. They suppose between 10-20% of the strokes in general (466).

They are divided according to their location and based on their pathophysiology into deep or lobar ICH . Deep ICH are produced by hypertensive arteriolosclerosis and lobar ICH are produced by CAA **[Table 5].**

The incidence clearly increases with age, especially in patients older than 85 years where a higher incidence is reached (467).

The most important risk factor is hypertension, the association has also been shown for lobar hemorrhages (468).

Other risk factors are smoking, alcohol intake with an approximate cut off 56 g/day , diabetes mellitus, and anticoagulant use (469–471).

The association between cholesterol and lipids with the risk of cerebral hemorrhage is controversial. Longitudinal studies have suggested that hypercholesterolemia is associated with a lower risk of ICH; however, other case-control studies have implicated high cholesterol levels as a factor of risk for ICH. The mechanism is unclear but it is believed that low cholesterol levels can produce a weak endothelial wall (470).

Hypertensive ICH develops in areas supplied by small perforating arterioles and prone to rupture to hypertension related pathologic-changes including lipohyalinosis, microaneurysm or fibrinoid necrosis (354).

The most frequent locations are in the basal ganglia (40%), the thalamus (10-15%), the cerebellum (5-10%), the pons (5%) and less frequently in the neocortex (20%) (472).

Neurologic symptoms are related to the location and the size of hematoma, mass effect from hematoma and brain edema after ICH (473).

CHARACTERISTICS	CEREBRAL AMYLOID ANGIOPATHY	SPORADIC NON-AMYLOID MICROANGIOPATHY "HYPERTENSIVE ARTERIOPATY¨
Small vessel pathology	Amyloid-B deposition and associated vasculopathy in cortical and leptomeningeal vessels	A range of different features, e.g. arteriosclerosis, fibrinoid necrosis, mural damage, etc
Intracerebral hemorrhage	Lobar (cortical-subcortical), cerebellar	Typically deep: basal ganglia, thalamus, pons, cerebellum; sometimes lobar
Ischemic stroke	Not typically associated with lacunes	Lacunar syndromes
Other clinical syndromes	Transiet focal neurological episodes (¨amyloid spells¨), cognitive impairment and dementia, inflammatory CAA	Vascular cognitive impairment and dementia
Cerebral microbleeds	Lobar	Deep and cerebellum
Cortical superficial siderosis	Very common: 40% in symptomatic CAA	Rare < 5%
MRI-visible perivascular spaces	Centrum semiovale (i.e. cerebral white matter)	Basal ganglia
White matter hyperintensities	Posterior predominance	No predilection for brain region

Table 5: Comparative table between the main radiological phenotypes of sporadic non amyloid angiopathy and sporadic CAA. Modified from (4).

JUSTIFICATION

In SVD or CMA there are four different clinical-radiological phenotypes often coincident, in fact, we have to consider SVD as a spectrum disorder. The phenotypes are leukoaraiosis (LA), microhemorrhages (MH), most lacunar infarcts (LI) and an unknown proportion of intracerebral hemorrhages.

Previously most research had focused on individual features of SVD and did not recognize the combined components as one disorder. In our study we have analyzed each phenotype separately but we have also analyzed the presence of any phenotype as a neuroimaging marker of the disease. To understand this disease, it would be interesting to analyze each phenotype separately.

Other important aspect is cognitive impairment in SVD. Cognitive components of microvascular damage in the brain have been mostly ignored, probably overshadowed by the attention given to AD but SVD actually is common in patients with AD, which suggest a synergistic or additive effect in the development of cognitive deterioration in these patients.

Previous studies has been focused in advanced stages of the disease, in patients who had suffered a lacunar stroke or in patients with history of ICH or with VaD, but there is a lack of studies that evaluate the factors that lead to the development of this disease in early stages. The causes of different phenotypes as well as the influence exerted by risk factors and the natural history of SVD is poorly understood. Since there are no early diagnostic markers and the therapeutic approach is empirical and totally inadequate, studies are needed to clarify these issues.

The pathophysiological aspects that lead to the development of SVD is not well understood. How vascular risk factors can influence the cerebral blood vessels producing microangiopathy, the possible role of breakdown of the BBB, particularly the effect of MMPs and the influence of endothelial dysfunction is unknown.

It would be interesting the better understanding of the natural history of the SVD to determine early diagnostic markers to anticipate these events and discover a risk profile to intervene and stop the process of vascular and neurodegenerative damage, thus avoiding future events that leave serious sequelae.

OBJECTIVES

- Main objective: Identify the clinical risk profile associated with the development of any SVD phenotype.

- Secondary objectives:

    - Identify biomarkers of cognitive impairment (Aβ 1-40), endothelial dysfunction (sTWEAK) and extracellular matrix dysfunction (MMPs) associated with initial phases or with the development of any phenotype.
    - Identify ultrasonographic and neuroimaging markers associated with initial phases or with the development of any of SVD phenotype.

MATERIAL AND METHODS

We developed a prospective study, selecting a high-risk population without known small vessel disease with high risk of suffering . We included patients over than 60 years old with a diagnosis of arterial hypertension and/or diabetes mellitus with more than five years of evolution, without history of neurological or severe systemic disease. The follow-up time was more than one year in each case.

Patients were selected in primary care and subsequently referred to the University Clinical Hospital of Santiago de Compostela where they were evaluated by neurologists, neuropsychologists and neuroradiologist of the center.

The **Local Ethical Committee of the Clinical University Hospital of Santiago de Compostela (Santiago-Lugo)** approved the protocol. The **code of registration is 2016/399.** All patients signed an informed consent.

This study was supported by the Instituto de Salud Carlos III (PI 13/02027: Estudio predictivo del desarrollo de la enfermedad de pequeño vaso cerebral en pacientes de alto riesgo).

The author and the directors of the work agreed to present the results in this Thesis and declare no conflict of interest.

Clinical, neuro-radiological, cognitive, ultrasonographic and molecular variables were included in a database to study what clinical risk profile and other variables are associated with the progression of small vessel disease. The cognitive, molecular and ultrasonographic variables were analyzed at baseline and every year. The radiological variables were analyzed at baseline and after two years.

The main variable of the study is the appearance or progression of any of the SVD phenotypes during the follow up: leukoaraiosis (LA), microhemorrhages (MH), lacunar infarcts (LI) and cognitive impairment. The secondary variables of the study are the appearance or increase of each one of the SVD phenotypes during the study follow-up time.

Clinical, neuroradiological and laboratory staffs were blinded respect to each others data.

1. SUBJECT SELECTION

1.1. PATIENT SELECTION

We prospectively included patients with hypertension (HT) and or diabetes mellitus type 2 (DM2) that met all the following inclusion criteria and none of the exclusion criteria.

1.1.1. Inclusion criteria

- Age between 60-75 years.
- History of HT and / or DM2

We defined hypertension and diabetes mellitus type 2 according the currently guidelines (HT ≥ 140/90 mmHg and HbA1C ≥ 6.5%, glycemia ≥ 200 mg / dl in symptomatic patients, baseline glycemia ≥ 126mg / dl in two determinations or glycemia after oral glucose tolerance test (OGTT) ≥ 200 mg / dl) of more than 5 years of evolution.

1.1.2 Exclusion criteria

- Previous history of transient ischemic attack, ischemic stroke or ICH.
- History of vascular disease: symptomatic peripheral arterial disease, coronary disease (history of angina, coronary revascularization or acute myocardial infarction (AMI)), advanced retinopathy (hemorrhage, exudates and papilledema in the context of proliferative diabetic retinopathy or malignant

hypertensive retinopathy), nephropathy, that is, diabetic nephropathy or deterioration of renal function (plasma creatinine> 1.5 mg / dl in men,> 1.4 mg / dl in women).
- Previous diagnosis of cognitive impairment or dementia
- Embolic heart disease (atrial fibrillation, atrial flutter, dilated cardiomyopathy)
- Cancer or severe systemic disease with a life expectancy less than 5 years.
- Chronic inflammatory disease (vasculitis, connective tissue diseases, inflammatory central nervous system diseases, intestinal inflammatory diseases, pelvic inflammatory disease, psoriasis, venous ulcers or chronic obstructive pulmonary disease).
- Inclusion in other clinical trial.
- Refuse of the patient to participate in the study.

1.2 FOLLOW-UP INTERRUPTION

Once included, patients will be followed for at least 1 year according to the visits summarized below.

Patients who present any of the following characteristics will be removed from the study:
- Withdrawal of informed consent
- Appearance of some of the following events:
    - Cardiac: angina, coronary revascularization or AMI
    - Neurological: TIA, ischemic stroke, ICH, dementia.
    - Peripheral vascular: intermittent claudication, arterial ischemia, abdominal aortic aneurysm, arterial revascularization.
    - Ophthalmological: proliferative retinopathy or malignant hypertensive retinopathy.

During the follow-up, the clinicians who evaluated the different risk markers were blinded to the control of the risk factors carried out by the primary care physicians. In the case that an event justifying the interruption of the follow-up was detected, patients were notified by

means of a report to his doctor so that they could leave the study and start the pertinent treatment.

1.3 PATIENT EVALUATION

All patients were selected by Primary Care physicians from Porto Do Son and A Estrada in the province of A Coruña and Pontevedra (Spain) respectively. Once the patient was selected was referred to the University Clinical Hospital of Santiago de Compostela.

Patients were revised every 6 months in primary care and once a year in the University Clinical Hospital.

Evaluation of patients in Primary Care at each visit included:

1. Age
2. Gender
3. Demographic and anthropometric data: weight, height, abdominal perimeter, body mass index (BMI).
4. Medical history and usual treatments were collected; data about history of DM2, HT, dyslipidemia (DLP), smoking and history of alcohol consumption. The degree of control of risk factors throughout the follow-up has also been analyzed.

In the parameters in relation to HT, good clinical control of HT in the follow-up was defined as blood pressure in the medical consultation less than 140/90 mmHg the 50% of the times that the patient was revised; bad clinical control of HT in the follow-up was defined as more than 140/90 mmHg the 50% of the times that the patient was revised.

We also took into account the good ambulatory BP monitoring (ABMP) control as blood pressure mean less than 130/80 mmHg (American Heart Association values), and good control of blood pressure in diabetics in the follow up as less than 130/80 mmHg the 50% of the times that the patient was revised; white coat effect was considered when the patient presented bad clinical control but good control in ABMP. Data of the of type antihypertensive treatment as well as the number of drugs were collected.

Resistant hypertension was considered if the patient was treated with 3 or more antihypertensive but the values remained above 140/90 mmHg.

The degree of control of DM2 was defined based on the 2015 ADA guidelines; in those under 65 years old without complications of diabetes the goal of glycosylated hemoglobin (HbA1c) is < 7% and if there are complications of more than 15 years of evolution of diabetes the goal is < 8%. In patients between 66 and 75 years without complications and with less than 15 years of evolution the goal of HbA1c is < 7% and if more than 15 years and with complications or serious comorbidities < 8,5%. In patients older than 75 years the goal is < 8,5%. Good control of DM2 in the follow-up was defined as more than 50% of the time with good control (reaching the objectives), bad control of DM2 in the follow-up was defined as less than 50% of the time with bad control. Data of type of antidiabetic treatment or insulin therapy were collected.

Dyslipidemia (DLP) was defined by sem FYC guidelines of 2012 and considered levels of total cholesterol > 250 mg/dl or LDL > 130 mg/dl or uptake of statin therapy. In diabetic patients a diagnostic of dyslipidemia was defined by levels of total cholesterol > 200 mg/dl.

The degree of control of DLP was defined based on the prevention guidelines of ESC in relation to cardiovascular risk. In patients with low or moderate cardiovascular risk the goal of LDL is < 115 mg/dl, in patients with high risk the goal of LDL is < 100 mg/dl and patients with very high risk the goal of LDL is < 70 mg/dl. Good control of DLP in the follow-up was defined as more than 50% of the time with good control (reaching the objectives) and bad control as less than 50% of the time with bad control. Data of statin therapy were collected.

5. Blood sample collection for blood count, biochemistry and coagulation test. Specifically HbA1c, lipid profile, renal function with albuminuria, erythrocyte sedimentation rate (ESR) and fibrinogen were analyzed.
6. 12-lead electrocardiography (ECG) was realized each visit to exclude patients with atrial fibrillation or atrial flutter.

7. ABMP was realized once a year to determine the control of blood pressure, goal blood pressure during activity is < 135/85 mmHg, during sleeping goal is < 120/70 mmHg and in 24 hour period goal is < 130/80 mmHg and the nocturnal hypertension is defined as patterns non-dipper expressed by a percentage difference between blood pressure in sleep and waking blood pressure less than 10%.

At the hospital at each visit data collected were:

1. Cognitive evaluation: Mini-Mental State Examination (MMSE), Addenbrooke`s Cognitive Examination (ACE) and Geriatric Depression Scale (GDS) of Yesavage were performed.

   The version used in this research is an adaptation to a rural community in Galicia, since the original ACE was aimed at subjects with a high educational level. In this version, due to the characteristics of this population, the name of the president of the United States was replaced by that of the Pope and the reading of irregular words was adapted (447).

   The cut-off used to classify the subjects as cognitive dysfunction / non-cognitive dysfunction, is the one proposed by Caballero et al for the Galician population: 65/100 (with a sensitivity of 90% and a specificity of 83%) for subjects with low educational level (finishing the studies before the age of 14) and 74/100 (with a sensitivity of 96% and a specificity of 85) %) for patients with medium and/ or high educational level.

   In this evaluation, scores equal or less than 74 points in patients with schooling over the age of 14 were considered pathological, scores equal or less than 68 points in patients with schooling below the age pf 14 were considered pathological. It was also analyzed the score obtained in MMSE and those bellow 24 points were considered pathological.

   Profile of cognitive impairment was obtained with the coefficient VLOM, with values over 3,2 were suggestive of AD, values below 2,2 were suggestive of subcortical profile and values between 3,2 and 2,2 were considered indeterminate.

Progression of cognitive impairment was defined as the sustained decrease in the score (previously considered pathological) of the tests performed in subsequent visits.

To evaluate depression, we used the GDS of Yesavage adapted to Spanish. It is a widely used and tested instrument, in different contexts, to evaluate depression in elderly patients. It can be applied in subjects who do not present any health problem, who have a medical illness or who even present a cognitive impairment that is not serious. It has a sensitivity of 92% and a specificity of 89% to identify adults with depressive symptomatology (474)

GDS encompasses key aspects of depression, evaluating cognitive symptoms, motivation, self-image, future and orientation to the past. It does not cover somatic aspects because it does not yield clinically significant information on depression in old age.

It is constituted by a test with 30 true / false questions; 20 of these questions indicate the presence of depressive symptomatology when responding positively and the rest when responding negatively. The questions of greatest discrimination are 3, 4, 5 and 8. The cut-off point for the diagnosis of depression is 15 points, a 12cut-off point is also normally use to determine a possible depression.

2. Neuroradiological evaluation by MRI:

An MRI of 1,5 Tesla was used. All studies were performed with the same MRI model.

Subjects were excluded from MRI examination if they had metal in the eyes or central nervous system, claustrophobia, valvular prosthesis, cardiac pace-marker, vascular clip, cochlear implant or other implantable devices, or if they refused. These patients were included in the study but not in the neuroimaging section.

To observe the presence and severity of leukoaraiosis we used Fazekas scale: grading 1,2 and 3.

IL was defined as CSF containing cavity, between 3 mm and 15 mm diameter in FLAIR image or T1/T2 or in sequences of DWI as acute small subcortical infarct (between 3 mm and 15 mm).

MH were defined as rounded and homogenous areas of signal loss < 10 mm in diameter in T2*. We discarded images suggestive of artifacts such as symmetrical areas of physiological calcification in the globus pallidus, small cavernous hemangiomas and partial volumes from vessels in the cortical sulcal. Presence, number and location of MH were determined.

MRI scans were observed by three neurologists blinded to clinical history.

3. Doppler ultrasonography evaluation in carotid and intracranial vessels to assess intima-media thickness (IMT), to observe the presence of atheroma plaques and hemodynamic stenosis and to assess pulsatility and resistance index in the media cerebral artery (PI and RI).

2. STUDY VARIABLES

2.1. PRIMARY ENDPOINT

The main variable of the study is the *progression of any SVD phenotypes* defined as appearance or increase of LA, LI, MH and cognitive impairment during the follow-up.

Variable *some marker of SVD,* was analyzed at basal study; this variable includes the presence of LA and/or presence of LI and/or presence of MH and/or presence of cognitive impairment.

2.2 SECONDARY ENDPOINTS

The secondary variables of the study are the appearance or increase of each one of the SVD phenotypes during the study follow-up period.

1. Progression of cognitive impairment

2. Progression of leukoaraiosis
3. New lacunar infarcts
4. New microhaemorrhages.

2.3 LABORATORY TEST

ELISA tests were performed to determinate the serum concentration of the following molecules: sTWEAK, Aβ1-40, TIMP1 and also the metalloproteinases MMP-1, MMP-10, MMP-7, MMP-9, MMP-12, MMP-13, MMP-3.

To determinate the concentration of TWEAK, Aβ1-40 and TIMP1 an Enzyme-Linked ImmunoAbsorbent Assay (ELISA) kit of *Elabscience* was used, with a technique of Sandwich-ELISA technique. First, we predicted the concentration before assaying, obtained the sample concentration in the range of the standard curve, determining the optimal sample solutions. If the dilution was necessary the procedure consisted in adding 500 uL of Reference Standard & Sample Diluent to each tube and pipette 500 uL of the 10 ng/mL working solution to the first tube and mix up to produce a 5 ng/mL working solution.

Sandwich-ELISA quantifies antigen concentration between two layers of antibodies: the first antibody, known as capture antibody, is precoated to the micro-ELISA plate. The second antibody, known as detection antibody, is added during the process. The detection antibody is biotinylated and, because of this, it binds specifically in a nearly irreversible manner to Avidin-Horseradish Peroxidase (HRP), which gives a signal depending on the concentration of the antigen. Only those that contain the molecules (sTWEAK, Aβ1-40 and TIMP1) bound to biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. Optical density (OD) is then measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of the

molecules of the study in the samples by comparing the OD of the samples to standard curve.

sTWEAK, Aβ1-40 and TIMP-1 kits were stored at a temperature of 4º for less than one month.

No freeze-thaw cycles were used and all reagents were brought to room with temperature between 18-25ºC.

Brief summary of the experiment operation:

Reagent preparation:

1. Take the kit out from the refrigerator 20 minutes in advance to bring all reagents to room temperature (18-25ºC) before use.
2. Bring the samples to room temperature (18-25ºC) before use, mix fully, avoid foaming.
3. Dilute the 25x concentrated wash buffer to 1x working solution
4. Dilute Reference Standard to different concentrations.
5. Dilute the 100x Biotinylated Detection Ab to 1x working solution 15 minutes earlier before step 1 finished.
6. Dilute the 100x Concentrated HRP Conjugate to 1x working solution 15 minutes earlier before step 2 finished
7. Preheat the microplate when step 6 starts.

Assay procedure:

1. Add 100 uL of standard or sample to each well. Incubate for 90 minutes at 37ºC
2. Remove the liquid. Add 100 uL of Biotinylated Detection Ab. Incubate for 1 hour at 37ºC.
3. Aspirate and wash 3 times
4. Add 100 uL of HRP Conjugate. Incubate 30 minutes at 37ºC
5. Aspirate and wash for 5 times
6. Add uL of Substrate Reagent. Incubate for 15 min at 37ºC
7. Add 50 uL of stop solution. Determine the OD value at 450 nm immediately
8. Calculation of results

To determinate the concentration of MMP-3, MMP-12 and MMP-13 an ELISA kit of Human Premixed Multi-Analyte Kit of *Luminex* was used, with a technique of Multiplex for simultaneous detection of multiple human biomarkers in serum. This system is used to analyze the levels of several biomarkers in a single sample. For ease of use, the microparticles are premixed in one vial as are the biotinylated detection antibodies.
The Magnetic Luminex principle consist of analyte-specific antibodies that are pre-coated onto magnetic microparticles embedded with fluorophores at set ratios for each unique microparticle region.

Microparticles, standards and samples are pipetted into wells and the immobilized antibodies bind the analytes of interest. After washing away any unbound substances, a biotinylated antibody cocktail specific to the analytes of interest is added to each well.

Following a wash to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (Streptavidin-PE), which binds to the biotinylated antibody, is added to each well. Final washes remove unbound Streptavidin-PE, the microparticles are resuspended in buffer and read using the *Luminex MAGPIX Analyzer.*

A magnet in the analyzer captures and holds the superparamagnetic microparticles in a monolayer. Two spectrally distinct Light Emitting Diodes (LEDs) illuminate the microparticles.

One LED excites the dyes inside each microparticle to identify the region and the second LED excites the PE to measure the amount of analyte bound to the microparticle. A sample from each well is imaged with a couple-charged device (CCD) camera with a set of filters to differentiate excitation levels.

The storage of the kits of MMP-13, MMP12, MMP13 was at a temperature of 2-8ºC for less than one month.

The serum samples were stored at < -20ºC.

No freeze-thaw cycles were used and all reagents were brought to room with temperature between 18-25ºC.

Brief summary of the experiment operation:

<u>Reagent preparation:</u>

1. On the day of the assay, previously frozen serum samples require centrifugation at 16,000 xg for 4 minutes immediately prior to use or dilution.
2. To determine the appropriate dilution for each analyte, refer to the table located in the link of the RD systems a bio techne brand web.
3. Dilute microparticle cocktail preparation using Diluent RD 2-1 in the mixing bottle provided.
4. Dilute biotin-antibody cocktail preparation in Diluent RD2-1.
5. Streptavidin-PE preparation: using a polypropylene amber bottle or a polypropylene test tube wrapped with aluminum foil. Protect the Streptavidin-PE from light during handling and storage.

Assay procedure:

1. Add 50 uL of standard or sample per well. A plate layout is provided to record standards and samples assayed.
2. Resuspend the dilute Microparticle Cocktail by inversion or vortexing. Add 50 uL of the microparticle cocktail to each well of the microplate. Securely cover with a foil plate sealer. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker set at 800 ± 50 rpm.
3. Using a magnetic device designed to accommodate a microplate, wash by applying the magnet to the bottom of the microplate, allow 1 minute before removing the liquid, filling each well with Wash Buffer (100 uL) and allow 1 minute before removing the liquid again. Complete removal of liquid is essential for good performance. Perform the wash procedure three times.
4. Add 50 uL of diluted Biotin-Antibody Cocktail to each well and Incubate for 60 minutes at room temperature on the shaker at 800± 50 rpm.
5. Repeat the wash.
6. Add 50 uL of diluted Streptavidin-PE to each well and incubate for 30 minutes at room temperature on the shaker at 800± 50 rpm.
7. Repeat the wash.
8. Resuspended the microparticles by adding 100 uL of wash Buffer to each well. Incubate for 2 minutes on the shaker set at 800 ± 50 rpm.

9. Read within 90 minutes using a Luminex or Bio-Rad analyzer.
10. Calculation of results.

2.4 STATISTICAL METHOD

2.4.1. Sample size

Calculation of sample size was made according to the following data. It is estimated that 30% of the hypertensive population over 60 years of age, with more than 5 years of evolution of hypertension, has LA in MRI brain studies. LA progresses in 3 years, (increasing the severity of Fazekas scale) between 40 and 70% of cases, therefore a sample size between 90 and 130 patients is needed to obtain a statistical power of 80% with a significant difference level of 0,05. *Epidat* software was used.

2.4.2 Statistical analysis

The statistical analysis was performed using the statistic program IBM®SPSS® statistics v.20 for Mac.

To identify the variables that followed a normal distribution, the Kolmogorov-Smirnov test was used. Continuous variables with normal distribution were expressed as mean (SD) and those variables not normally distributed were expressed as median [quartiles].

Proportions between groups were compared by chi-square test. Student's T test was used to compare continuous variables with normal distribution between 2 groups. In case of variables with no-normal distribution, Mann-Whitney U test was used to compare 2 groups. In case of more than 3 groups, variables were compared using ANOVA test.

In order to assess variables that were independently associated with progression of SVD, progression of cognitive impairment, progression

of LA, new LI and new MH, a multivariate logistic regression analysis was performed. Odds ratios were adjusted by significant variables in the bivariate analysis. The results were expressed as adjusted odds ratios (OR) with corresponding 95% confidence intervals (95% CI).

Values of p below 0.05 were considered to be statistically significant in all tests.

RESULTS

1. BASAL CHARACTERISTICS OF PATIENTS

207 patients were evaluated between 2013 and 2016 with history of hypertension and/or diabetes mellitus of more than 5 years of evolution, independently of the degree of control.

106 patients were excluded for presenting some exclusion criteria; 85 patients refused to participate, 12 patients were in atrial fibrillation in basal ECG and 9 had deterioration in renal function in basal analysis.

The number of patients included was one hundred and one (101). During the follow up, 9 patients were lost; 6 of them did withdrawal of informed consent and 3 of them developed an exclusion criteria (one of them developed dementia, other one suffered peripheral artery disease and other patient suffered a stroke). Mean time of follow up was 24 ± 4,86 months.

59,4% were women. Mean age was 71,02 ± 5,24 years.

Mean height was 158±54 centimeters, mean weight was 76,74±13,85 kilograms and mean body mass index (BMI) was 30,49±4,62 kg/m^2 .

Regarding classification of BMI: 7,9% of the sample had normal weight , the 45,5% were overweight, the 28,7% were obesity type I, 11,9% obesity type II and 5,9 were obesity type III **(Figure 1).**

Mean waist perimeter was 101,96 cm ± 12,31 cm.

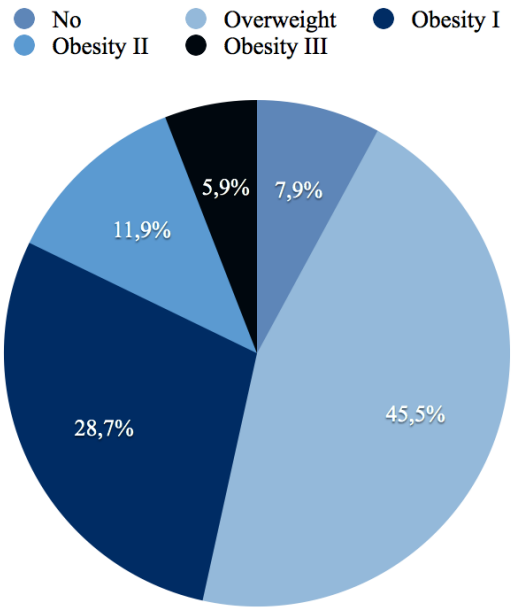


Figure 1. Basal percentage of obese according to IBM.

At the time of inclusion 99% of the sample was hypertensive, of which 42,6% had good clinical control and 57,4% bad clinical control.

Regarding antihypertensive treatment, 3% did not receive any treatment, 32,6% received one antihypertensive drug, and 64,4% of hypertensive patients received more than one antihypertensive drug.

According to the type of antihypertensive drug, 34% of the patients were treated with diuretic, 4% with betablockers, 4% with calcium antagonist, 24% with angiotensin-converting enzyme inhibitors and 29% with angiotensin-receptors blockers **(Figure 2).**

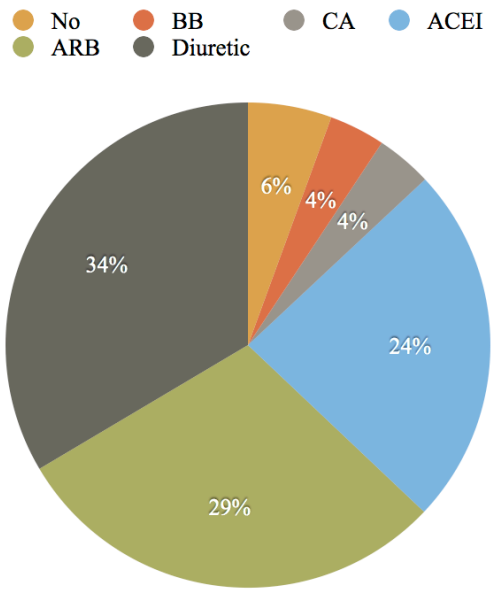


Figure 2. Basal percentage of patients with a certain type of antihypertensive.

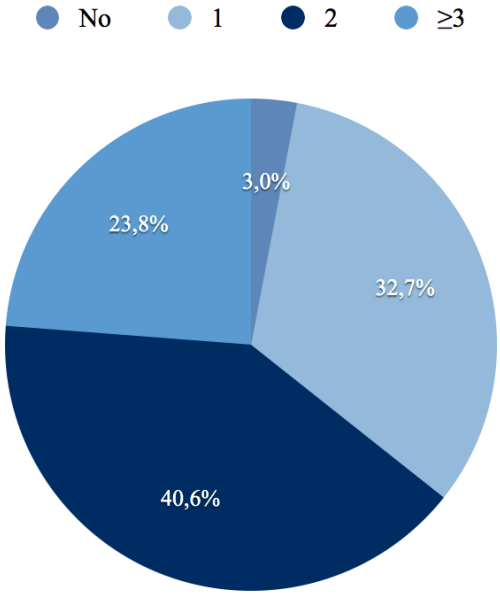


Figure 3. Basal percentage of patients with number of antihypertensive treatments.

79,2% had good control of blood pressure in ABMP and 20,8 % did not. ABMP was realized in 92 patients. Regarding nocturnal blood pressure profiles 55,4% of patients had dipper hypertension and 44,6% had non-dipper hypertension. White coat effect was detected in 33,3% of the sample. 23,8 % were treated with 3 or more drugs **(figure 3)**

Resistant hypertension was detected in 11,9% of the sample.

At the time of inclusion 36,6% of the sample had diabetes mellitus type 2, of which 54,3% had good glycemic control and 45,7% poor glycemic control. The average values of HbA1c (taking the maximum value during the follow up) of the sample registered during the follow-up were 7,7 %. The average values during the follow up annually is represented in **table 1.**

94,6% of the sample were treated with one or more oral antidiabetics of which metformin was 95%. Insulin therapy was administered a 27% of the sample.

	Basal	12 months	24 months
HbA1c	7,4%	7,1%	7,06%

Table 1. Average values of HbA1c of the diabetic patients during the follow-up.

74,3% of the sample had dyslipidemia at inclusion of which 65,4% had good control and 34,6% bad control. 71,3% of the sample was treated with a statin. The average values of cholesterol during the follow-up annually is represented in **table 2.**

	Basal	12 months	24 months
Total cholesterol (mg/dl)	194,9 (235-362)	184,7 (107-305)	182,6 (117-244)
LDL (mg/dl)	112,45 (51-263)	106,9 (25-210)	105,5 (58-164)
HDL (mg/dl)	59,2 (30-119)	57,9 (31-124)	54,07 (27-137)

Table 2. Average values of cholesterol and lipoproteins of the sample during the follow-up.

Referring to toxic habits, 9,9% of the sample had history of moderate alcohol consumption and 11,9% had history of smoking.

Respecting carotid ultrasonography variables, a 43,6% of the sample had carotid plaques in the follow-up, a 61,3% had pathological intima media thickness a 73,3% had pathological carotid pulsatility index and a 57,4% had pathological carotid resistance index.

Basal percentage of patients with abnormal carotid or intracranial ultrasound patterns is represented in **Tables 3-4**.

Carotid ultrasonography variables	Patients (%)
Pathological IMT	61,4
Pathological PI	73,3
Pathological RI	57,4
Carotid plaques	43,6

Table 3. Basal percentage of patients with abnormal carotid ultrasound patterns.

Respecting intracranial ultrasonography variables, a 46,6% of the sample had a pathological middle cerebral artery pulsatility index and a 24,8% had a pathological middle cerebral artery resistance index.

Intracranial ultrasonography variables	Patients (%)
Pathological PI	73,3
Pathological RI	57,4

Table 4. Basal percentage of patients with abnormal transcranial ultrasound patterns.

In relation to laboratory variables, including main molecules of research we show the average values of the sample during the 3 follow-up moments **(Table 5-6).**

	Basal	12 months	24 months
Leukocytes	6984±1903	6992±1908	6902±1635
Neutrophils	3749±1486	3697±1388	3711±1302
Creatinine	0,77±0,23	0,78±0,24	0,81±0,25
Fibrinogen	382±74,5	404,9±78,1	420,4±56,5

Table 5. Average values of leukocytes, neutrophils, creatinine and fibrinogen of the sample during the follow-up.

The average values of ESR (taking the maximum value during the follow-up) of the sample registered during the follow-up was 14,96 (±10,2).

	Basal	12 months	24 months
s-TWEAK	6366,62 ±7764,87	3970,49 ±3933,03	5339,42 ±6780,86
TIMP-1	1343,31 ±727,95	1252,33 ±633,97	773,47 ±631
AB 1-40	40,75±24,35	47,18±34,86	34,01±29,68
MMP-1	3,29 ±2,03	3,36±1,56	2,97±1,45
MMP-7	4,19±2,76	3,56±1,57	3,23±2,79
MMP-9	16,67±12,72	15,22±12,10	15,11±11,43
MMP10	1,01±0,86	0,8±0,43	0,93±1,45
MMP-3	14,32±9,14	13,82±7,12	13,26±6,11
MMP-12	0,15±0,07	0,20±0,086	0,14±0,07
MMP-13	0,78±0,17	0,87±0,19	0,77±0,22

Table 6. Average values of main molecules of research of the sample during the follow-up.

Respecting neuroimaging features, basal phenotypes of SVD such as leukoaraiosis (LA), lacunar infarcts (LI), microhemorrhages (MH) and cognitive impairment were analyzed.

LA was detected in 87,1% of the sample. Fazekas grade I was detected in 56,4%, Fazekas grade II in 24,8% and grade III in 5,9% of patients **(Table 7).**

Variable	Patients (%)
Leukoaraiosis	
No	12,9
Grade I	56,4
Grade II	24,8
Grade III	5,9

Table 7. Basal phenotypes of the sample: Leukoaraiosis grading by Fazekas.

LI were detected at the first MRI (basal study) in 9,9% of the sample, none of them were symptomatic **(Table 8).** MH were detected at basal MRI in 10,9%, the location of the MH was in 40% of the cases in corticosubcortical regions and in basal ganglia in 60% of the cases **(Table 9-10).**

Variable	Patients (%)
Lacunar infarcts	
No	90,1
Yes	9,9

Table 8. Basal phenotypes of the sample: presence of lacunar infarcts.

Variable	Patients (%)
Microbleeds	
No	89,1
Yes	10,9

Table 9. Basal phenotypes of the sample: presence of microbleeds.

Variable	Patients (%)
Location of Microbleeds (basal)	
Cortical and subcortical	45,5
Basal ganglia and brainstem	54,5

Table 10. Basal phenotypes of the sample: location of microbleeds.

21,8 % of patients (22 patients) presented, in basal test, results compatible with cognitive impairment, none in the dementia range **(Table 11).**

Variable	Patients (%)
Cognitive impairment	
No	77,2
Yes	21,8

Table 11. Basal phenotypes of the sample: presence of cognitive impairment.

Respecting cognitive impairment profile using VLOM coefficient, a 15,9% of the sample at basal test had an AD profile and a 2,9% a subcortical dementia profile. In 6,9% of patients the profile was indeterminate with mixed characteristics **(table 12).**

Variable	Patients (%)
Cognitive impairment profile	
Alzheimer disease	15,9
Subcortical	2,9
Indeterminate	6,9

Table 12. Basal phenotypes of the sample: presence of cognitive impairment

Some marker of SVD was defined as the presence of LA and/or presence of LI and/or presence of MH and/or presence of cognitive impairment at basal studies. Some marker of SVD at basal studies was detected in 87,1% of the sample **(table 13).**

Variable	Patients (%)
Basal some marker of SVD	
No	12,9
Yes	87,1

Table 13. Basal phenotypes of the sample: presence of some marker of SVD

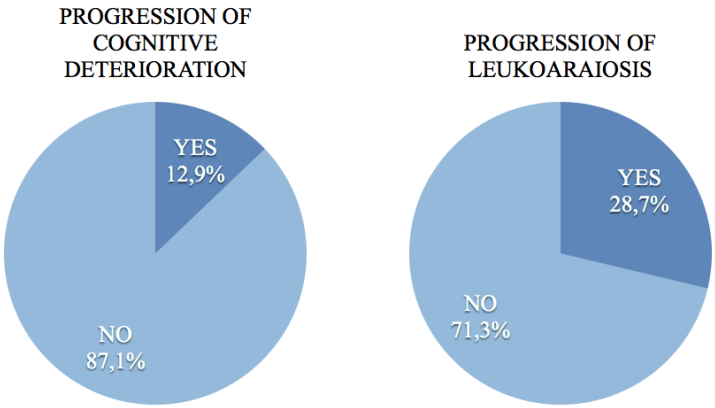
At basal examination **23,5% had depression** with punctuation in GDS of Yesavage test of more than 15 points. No patient without depression in the basal test developed depression.

2 . MAIN VARIABLE: PROGRESSION OF ANY SVD PHENOTYPE

2.1 DESCRIPTIVE ANALYSIS PROGRESSION OF ANY SMALL VESSEL DISEASE PHENOTYPE.

In relation specifically to appearance or increase of any of SVD phenotypes during the follow-up period, LA advance in 28,7% of cases (29 patients), new LI were detected in 14,9% (15 patients), new MH were detected in 14,9% of cases (15 patients) and cognitive impairment progresses in 12,9% of cases (13 patients).

Progression of any SVD phenotype defined as increase of LA and/or new LI and/or new MH and/or progression of cognitive impairment, were detected in 42,6% of cases (43 patients). None of patients presented symptomatic LI and none of them developed dementia **(Figure 4).**



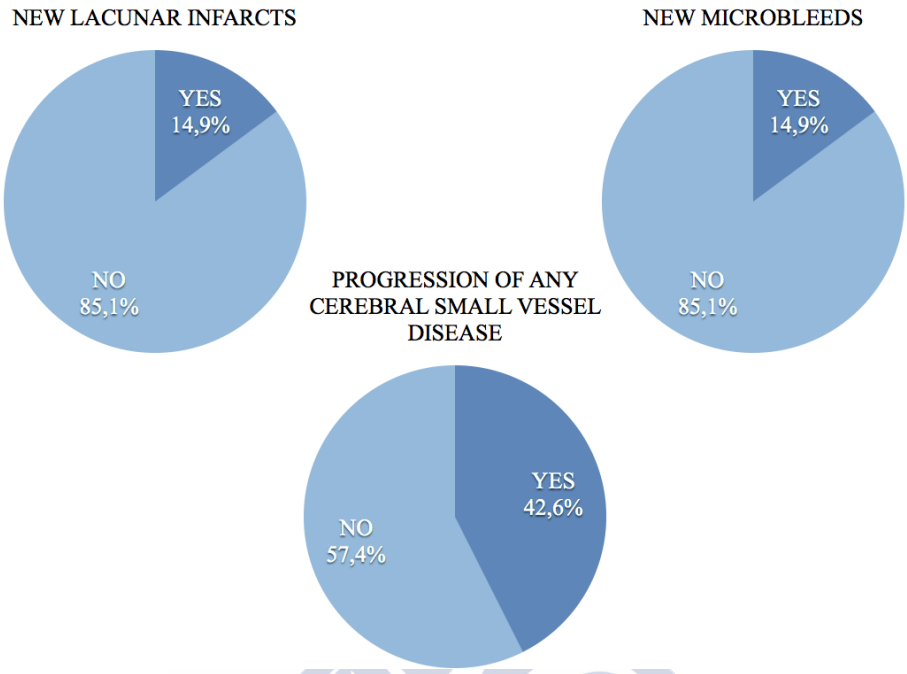


Figure 4: Representation of the percentage of progression of the main phenotypes and of any SVD phenotype (p.113-116).

2.2. BIVARIATE ANALYSIS OF THE MAIN VARIABLE: PROGRESSION OF ANY SMALL VESSEL DISEASE PHENOTYPE.

The bivariate analysis is used to determine which vascular risk factors may be associated with the progression of any SVD phenotype **(Table 14),** and which ultrasonographic alterations and which laboratory variables of habitual use are also found more frequently in patients with progression of the disease.

In patients with progression of any SVD phenotype, weight and BMI were lower. A greater number of patients in the group of

progression of any SVD had bad clinical control of blood pressure, hypertension of white coat and no-dipper pattern in ABMP; also, they had higher percentages of bad control of DLP than patients with no progression.

In the group with no progression we observed higher levels of BMI vs in the group of patients with progression of the SVD (31,3 ± 5,1 vs 29,3± 3,8).

Respecting ultrasonography variables, the group with progression had more carotid plaques and higher percentages of pathological resistance index in transcranial doppler.

Referring to laboratory variables ESR and fibrinogen at visit 3, levels were higher also in these group.

Variable	Progression of any small vessel disease Phenotype		
	No n= 58	Yes n=43	p
Age, years	71,3 ± 5,4	70,6 ± 5,1	0,496
Woman,%	51,7	48,3	0,113
Height, cm	159,7 ± 8,1	156,9 ± 7,8	0,192
Weight, Kg	79,9 ± 14,3	72,5 ± 12,1	0,008
BMI, Kg/m^2	31,3 ± 5,1	29,3 ± 3,8	0,031
Waist circumference, cm	103,4 ± 12,5	100,1 ± 11,9	0,177
Bad clinical control of HT, %	37,9	62,1	<0,0001
White coat effect, %	42,4	57,6	0,028
No-dipper %	44,2	55,6	0,015
Bad clinical control of DM2, %	31,2	68,8	0,108
DLP, %	53,3	46,7	0,118
Bad clinical control of DLP, %	33,3	66,7	0,008
Smoking, %	41,7	58,3	0,193
Alcohol consumption, %	60,0	40,0	0,570
ABI pathological, %	53,8	46,2	0,127
IMT pathological, %	61,3	38,7	0,216
IMT growth, %	0,07±0,07	0,07±0,07	0,530
Plaques, %	40,9	59,1	0,003
New plaques, %	66,7	33,3	0,414
Carotid PI pathological, %	51,4	48,6	0,033
Carotid RI pathological, %	50,0	50,0	0,060
Transcranial PI pathological, %	55,3	44,7	0,421
Transcranial RI pathological, %	34,1	65,9	<0,0001
Albumin-creatinine ratio (ACR)	56,03±201,05	48,53±46,44	0,999
ESR	13,67±8,67	21,88±12,16	<0,0001
Creatinine mg/dL, visit 3	0,94±0,85	0,77±0,24	0,019
Fibrinogen mg/dL, visit 3	389,19±69,14	442,26±68,6	<0,0001
Leukocytes x 10^3/mL visit 3	6,85±1,73	7,22±1,83	0,311
Neutrophils x 10 ^{3}/mL visit 3	3,67±1,45	3,83±1,33	0,558

Table 14. Bivariate analysis of the main variable: progression of any SVD phenotype

Respecting analysis of variance (ANOVA) graphs **(figure 5-8),** there is a trend in the evolution in the group in which progression of the SVD is observed to present higher levels of leukocytes and neutrophils although not statistically significant.

There are no differences in creatinine levels.

In relation to fibrinogen, a linear increase of the levels in the evolution is observed in the patients with progression, the differences observed in both groups are significant.

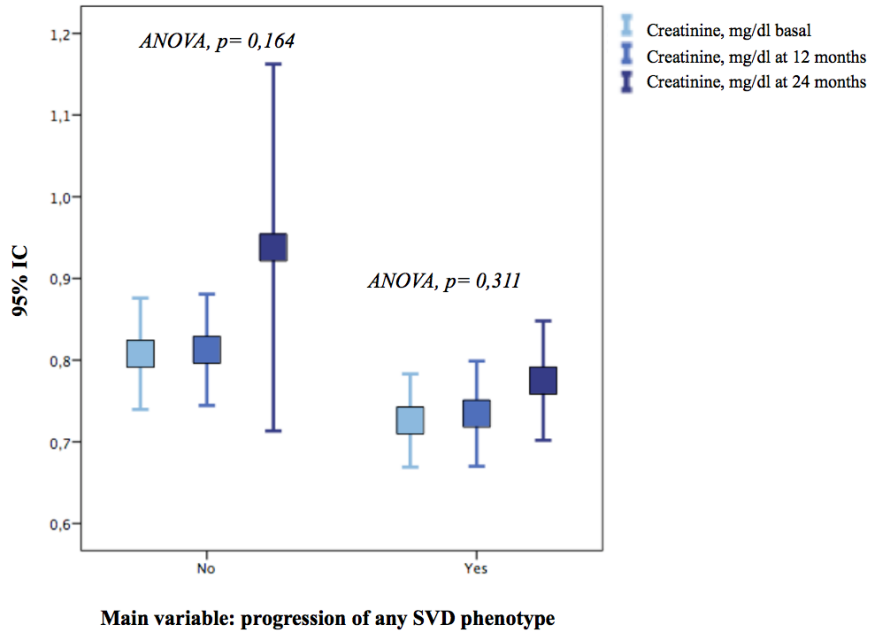


Figure 5: Evolution of levels of creatinine during the follow-up in patients with progression and without progression of any SVD phenotype.

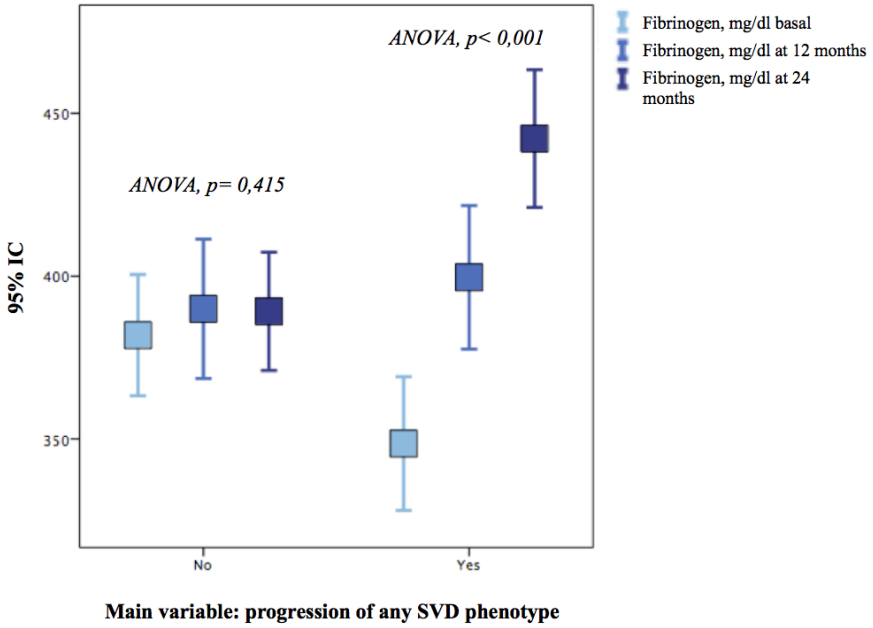


Figure 6: Evolution of levels of fibrinogen during the follow-up in patients with progression and without progression of any SVD phenotype.

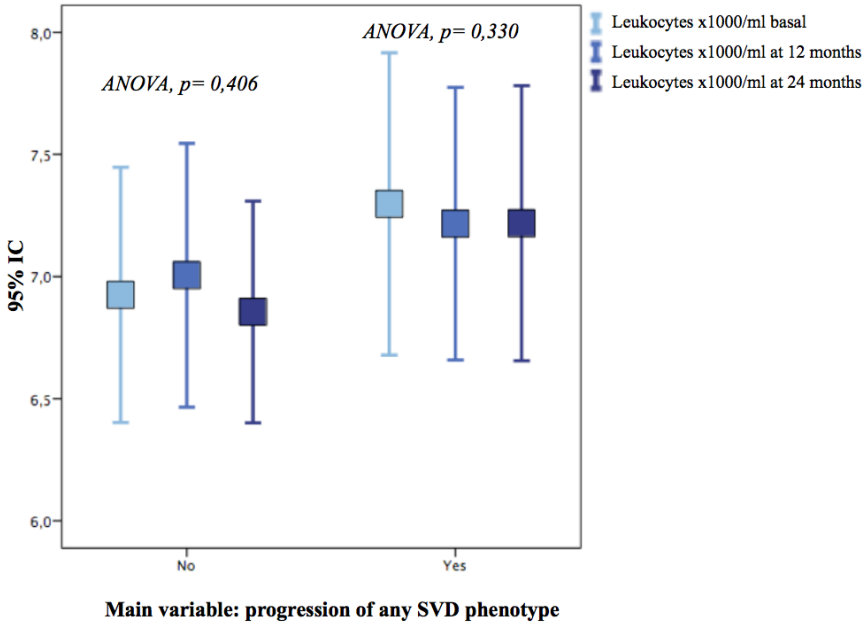


Figure 7: Evolution of levels of leukocytes during the follow-up in patients with progression and without progression of any SVD phenotype.

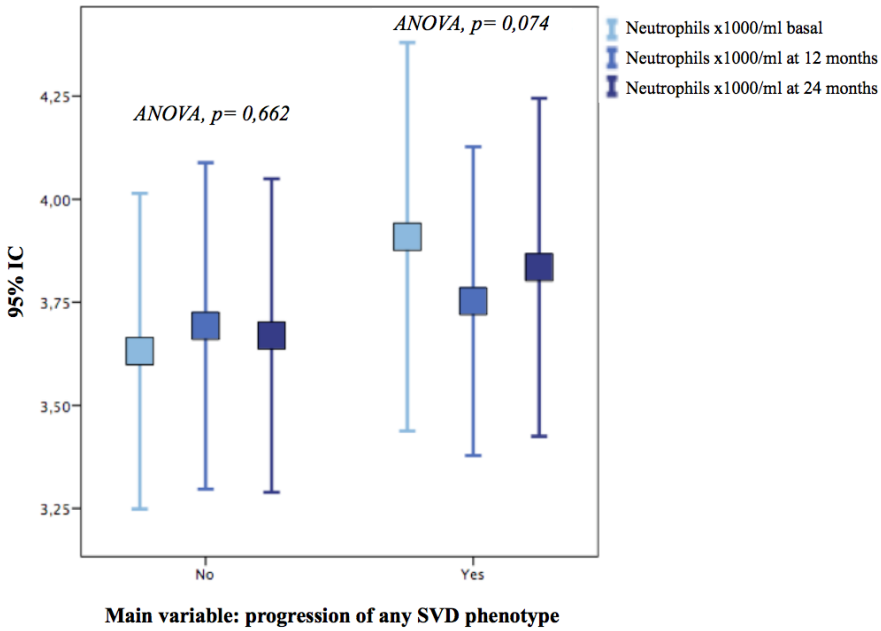


Figure 8: Evolution of levels of neutrophils during the follow-up in patients with progression and without progression of any SVD phenotype.

In relation to carotid and intracranial ultrasound parameters we observed in the group of progression higher percentage of patients with pathological levels of transcranial RI but we did not find differences in others parameters **(Figure 9).**

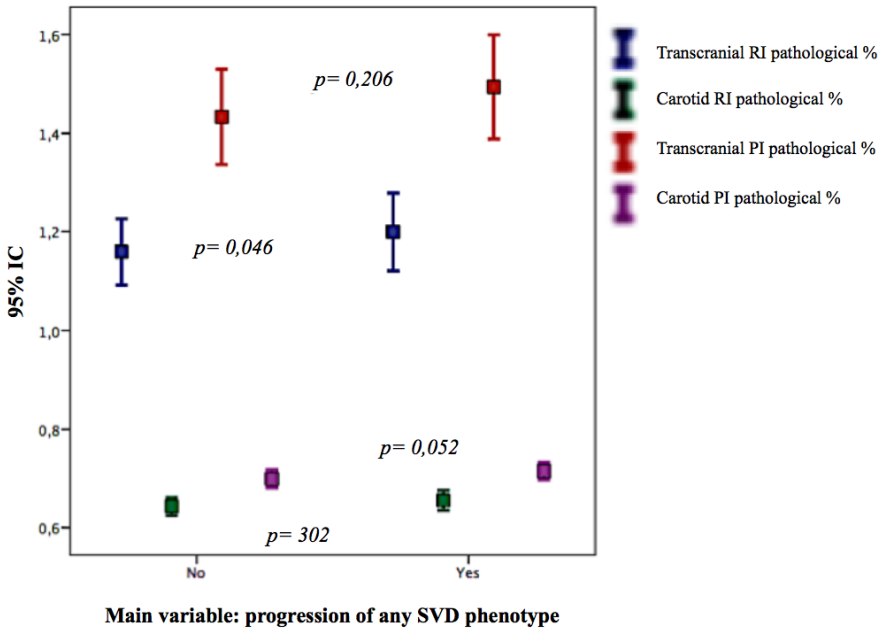


Figure 9: Differences in relation to ultrasonographic parameters in patients with progression and without progression of any SVD phenotype .

2.3 BIVARIATE ANALYSIS OF THE MAIN VARIABLE: PROGRESSION OF ANY SMALL VESSEL DISEASE PHENOTYPE WITH THE INCLUSION OF MOLECULAR MARKERS OF RESEARCH

The bivariate analysis is used to determine the association between the research molecules that have been selected with the progression of any SVD **(Table 15).**

In patients with progression of any SVD, levels of sTWEAK in basal samples, at 12 and 24 months were significantly higher than in patients in whom no progression was observed **(Figure 10).**

We have also observed that values of Aβ 1-40 in basal samples, at 12 and 24 months were higher in the group of patients with progression **(Figure 11).** Our sample has an average levels of Aβ 1-40 of

40,75±24,35 pg/ml at basal examination, 47,18±34,86 pg/ml at 12 months and 34,01±29,68 at 24 months.

In cases of progression of any phenotype of SVD levels of Aβ 1-40 was 75,2±46,2 pg/ml vs 36,9±23,3 pg/ml at basal test, 112,3±71,8 vs 52,1 ±28,9 pg/ml at 12 months and 107,0±82,4 vs 35,4 ±23,7 pg/ml. p 0,020, p < 0,0001 and p 0,001 respectively.

A tendency has been observed, but not statistically significant, to detect higher levels of MMP-7 and MMP-9 in the group of patients with progression of the disease.

	No n = 58	Yes n = 43	p
Basal TWEAK (pg/ml) TWEAK at 12 months TWEAK at 24 months	4732,5±2100,0 5064,2±2137,7 3398,4±1060,3	6885,9±2558,2 8810,2±4557,2 8442,2±4953,9	<0,0001 <0,0001 <0,0001
Basal AB 1-40 (pg/mL) AB 1-40 at 12 months AB 1-40 at 24 months	36,9±23,3 52,1±28,9 35,4±23,7	75,2±46,2 112,3±71,8 107,0±82,4	0,020 <0,0001 0,001
Basal MMP-1 (pg/mL) MMP-1 at 12 months MMP-1 at 24 months	3,2±1,8 3,9±2,4 3,1±1,6	3,0±1,4 3,2±1,4 3,6±1,9	0,496 0,923 0,163
Basal MMP-3 (pg/mL) MMP-3 at 12 months MMP-3 at 24 months	12,1±5,7 12,2±5,7 12,8±6,4	12,2±5,3 11,9±4,6 11,8±3,9	0,878 0,973 0,747
Basal MMP-7 (pg/mL) MMP-7 at 12 months MMP-7 at 24 months	3,8±2,0 3,0±1,1 2,3±0,9	7,3±4,1 6,4±3,2 8,3±6,7	0,224 0,088 0,425
Basal MMP-9 (pg/mL) MMP-9 at 12 months MMP-9 at 24 months	11,9±2,6 12,9±2,6 12,9±3,2	19,9±9,9 18,1±8,4 18,3±10,3	0,029 0,088 0,425
Basal MMP-10 (pg/mL) MMP-10 at 12 months MMP-10 at 24 months	0,8±0,5 0,7±0,3 0,6±0,4	1,2±0,9 1,0±0,7 0,7±0,3	0,392 0,075 0,425
Basal MMP-12 (pg/mL) MMP-12 at 12 months MMP-12 at 24 months	0,2±0,1 0,2±0,1 0,2±0,1	0,2±0,1 0,2±0,1 0,1±0,1	0,165 0,589 0,891
Basal MMP-13 (pg/mL) MMP-13 at 12 months MMP-13 at 24 months	0,8±0,2 0,9±0,2 0,8±0,2	0,7±0,1 0,8±0,1 0,7±0,1	0,165 0,589 0,891
Basal TIMP (ng/mL) TIMP at 12 months TIMP at 24 months	1004,4±269,9 1188,6±376,5 702,6±340,5	1443,1±900,1 1216,5±311,4 1040,3±968,0	0,111 0,684 0,386

Table 15: Bivariate analysis: plasmatic levels of the research molecules in patients with progression or not of any SVD phenotype.

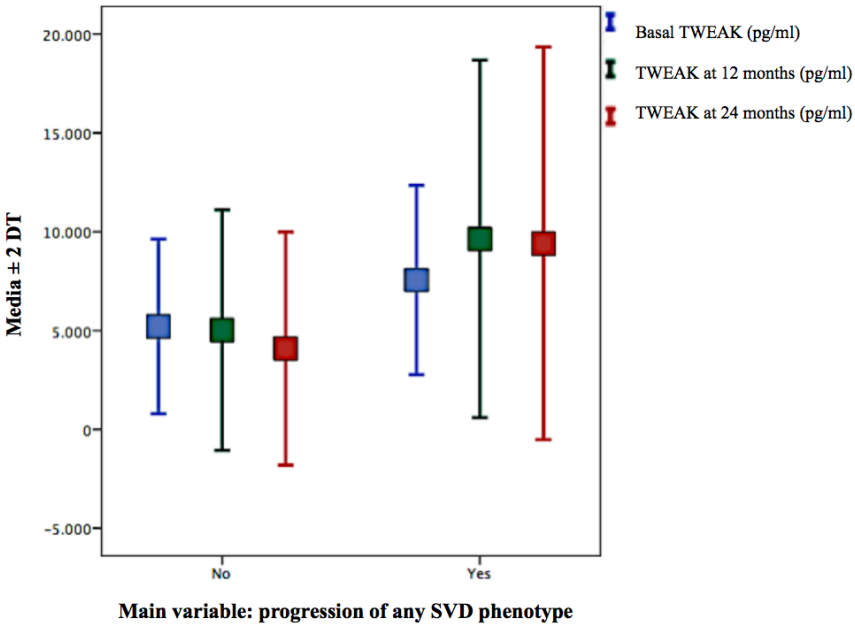


Figure 10: Differences in relation to sTWEAK levels in patients with progression and without progression of any SVD phenotype.

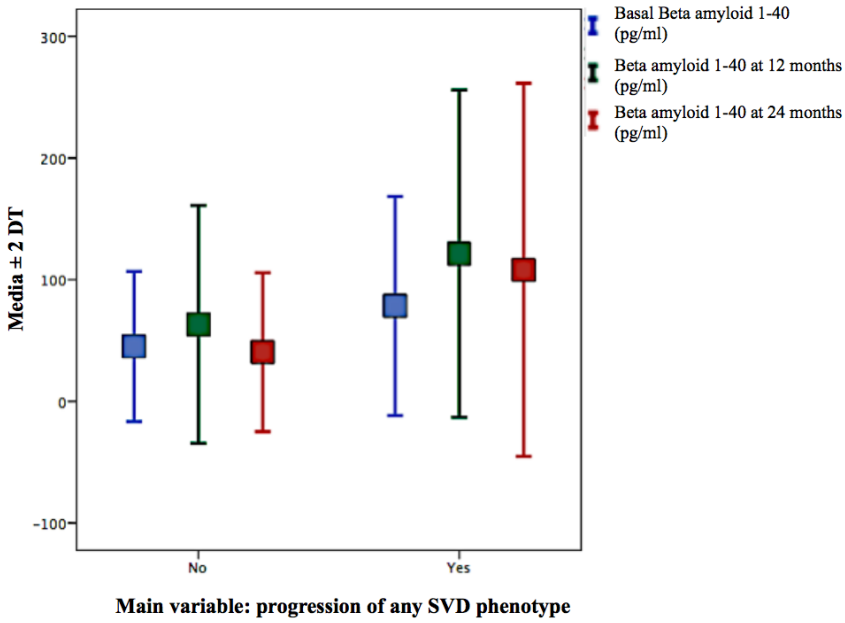


Figure 11: Differences in relation to AB 1-40 levels in patients with progression and without progression of any SVD phenotype .

2.4 MULTIVARIATE ANALYSIS OF THE MAIN VARIABLE: PROGRESSION OF ANY SMALL VESSEL DISEASE PHENOTYPE

In logistic regression models, we chosen variables with a significance < 0,001 in the bivariate model. Between the variables related to each other (for example, bad control of HT, no-dipper or white coat HT) they were selected those of greater statistical significance **(Table 16-17).**

In not adjusted model the variable that is more associated with progression of SVD is bad clinical control of HT (OR 8,42; 95% CI, 3,19-22,15); an association has also been found in patients with transcranial IR pathological (OR 5,30; 95% CI 2,23-12,57), bad clinical control of DLP (OR 3,75; 95% CI 1,37-9,82), higher levels of ESR (OR 1,12; 95% CI 1,06-1,18) and higher levels of fibrinogen during evolution or at visit 3 (OR 1,01; 95% CI 1,00-1,02). Patients with higher BMI had less progression of SVD and an inverse association was observed (OR 0,9; 95% CI 0,82-0,99).

In adjusted model the variable that is associated independently with statistical significance with progression of SVD is bad clinical control of HT(OR 4,7; 95% CI 1,18-18,91).

In the same way, in adjusted model, patients with higher BMI had less progression of SVD and an inverse association was observed (OR 0,84; 95% CI 0,73-0,97).

An association but without statistical significance has also been found in patients with pathological transcranial IR (OR 3,56; 95% CI 0,95-13,39), bad clinical control of DLP (OR 3,49; 95% CI 0,92-13,24), higher levels of ESR (OR 1,02; 95% CI 0,99-1,12) and higher levels of fibrinogen during evolution or at visit 3 (OR 1,01; 95% CI 1,00-1,02).

	OR*	CI (95%)	p
BMI	0,90	0,82-0,99	0,036
Bad clinical control of HT	8,42	3,19-22,15	<0,0001
Bad clinical control of DLP	3,75	1,37-9,82	0,010
Transcranial IR pathological	5,30	2,23-12,57	<0,001
ESR	1,12	1,06-1,18	<0,0001
Fibrinogen visit 3	1,01	1,00-1,02	0,001

Table 16: Multivariate analysis: influence of variables in the progression of any SVD phenotype. Crude Odds Ratio produced by a regression model.

	OR**	CI (95%)	p
BMI	0,84	0,73-0,97	0,018
Bad clinical control of HT	4,73	1,18-18,91	0,028
Bad clinical control of DLP	3,49	0,92-13,24	0,065
Transcranial IR pathological	3,56	0,95-13,39	0,059
ESR	1,02	0,99-1,12	0,071
Fibrinogen visit 3	1,01	1,00-1,02	0,038

Table 17: Multivariate analysis: influence of variables in the progression of any SVD phenotype. Adjusted Odds Ratio produced by a regression model.

2.5 MULTIVARIATE ANALYSIS OF THE MAIN VARIABLE WITH THE INCLUSION OF SIGNIFICANT MOLECULAR MARKERS OF RESEARCH IN THE BIVARIATE MODELS

In logistic regression models, we chosen variables with a significance < 0,001 in the bivariate model.

We selected significant molecular markers of research sTWEAK and Aβ 1-40 at 24 months because the most significant value found in the follow-up was used.

In not adjusted and adjusted models of higher levels of sTWEAK at 24 months the result of OR was 1 (CI 1,00-1,01) p 0,002 and 1 (CI 1,00-1,01) p 0,008 respectively, so we did not find that patients with higher levels of sTWEAK develop more likely progression of any phenotype of small vessel disease **(table 18-21).**

In the case of higher levels of Aβ 1-40 at 24 months in not adjusted and adjusted models the result of OR was 1,02 (CI 1,00-1,02) p 0,005 and 1,05 (CI 1,00-1,10) p 0,038 respectively, so we found that patients with higher levels of Aβ 1-40 develop with a discrete higher probability progression of SVD than patients with lower levels.

	OR*	CI (95%)	p
sTWEAK at 24 months	1,00	1,00-1,01	0,002

Table 18: Multivariate analysis: influence of levels of sTWEAK at 24 months in the progression of any SVD phenotype. Crude Odds Ratio produced by a regression model.

	OR**	CI (95%)	p
sTWEAK at 24 months	1,00	1,00-1,01	0,008

Table 19: Multivariate analysis: influence of levels of sTWEAK at 24 months in the progression of any SVD phenotype. Adjusted Odds Ratio produced by a regression model.

	OR*	CI (95%)	p
AB 1-40 at 24 months	1,02	1,00-1,03	0,005

Table 20: Multivariate analysis: influence of levels of AB 1-40 at 24 months in the progression of any SVD phenotype. Crude Odds Ratio produced by a regression model.

	OR*	CI (95%)	p
AB 1-40 at 24 months	1,05	1,00-1,10	0,038

Table 21: Multivariate analysis: influence of levels of AB 1-40 at 24 months in the progression of any SVD phenotype. Adjusted Odds Ratio produced by a regression model.

3. SECONDARY VARIABLES

3.1 PROGRESSION OF COGNITIVE IMPAIRMENT

3.1.1 Progression of cognitive impairment: descriptive analysis

Of the 22 patients who at the time of inclusion in the study had cognitive impairment, in 13 the clinical progressed (59,1%). Of patients with cognitive impairment in inclusion, 100% of those with a profile of AD progressed (7/7), as well as 66,7% of those with a subcortical profile (2/3), and 25% of those with indeterminate cognitive impairment (1/4) **(Figure 12).**

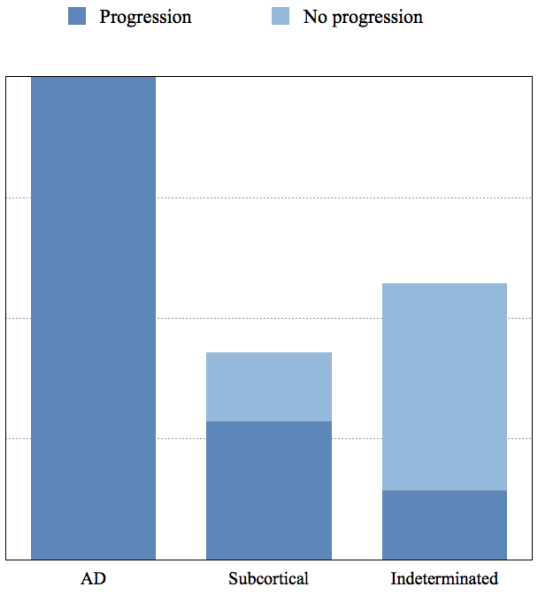


Figure 12. Profile cognitive impairment progression during the follow-up.

3.1.2 Progression of cognitive impairment: bivariate analysis

The bivariate analysis is used to determine which vascular risk factors may be associated with the progression of cognitive impairment and which ultrasonographic alterations, neuroimaging markers of SVD and which laboratory variables of habitual use are also found more frequently in patients with progression of cognitive impairment.

Despite checking some trend, no variable reached significant differences, probably in relation to small sample size of patients with cognitive impairment **(table 22).**

Variable	Progression of cognitive impairment		
	No n= 88	Yes n=13	p
Age, years	71,3 ± 5,9	73,1±4,3	0,525
Woman,%	22,2	23,1	0,684
Follow-up time, months	22,9±4,2	25,5±4,9	0,212
BMI, Kg/m^2	31,1±4,5	28,5±3,7	0,183
Bad clinical control of HT, %	55,6	69,2	0,416
Bad clinical control of DM2, %	33,3	71,4	0,303
DLP, %	25,0	41,7	0,392
Bad clinical control of DLP, %	33,3	66,7	0,008
Smoking, %	22,2	7,7	0,358
Alcohol consumption, %	11,1	7,7	0,662
ABI pathological, %	-	-	
New plaques, %	-	-	
Carotid IP pathological, %	55,6	76,9	0,276
Carotid IR pathological, %	44,8	61,5	0,361
Transcranial IP pathological, %	55,6	30,8	0,235
Transcranial IR pathological, %	33,3	61,5	0,193
Some neuroimaging marker to the inclusion %	100	84,6	0,338
Progression of LA, %	22,2	46,2	0,246
New LI,%	22,2	23,1	0,684
New MH, %	11,1	15,4	0,642
ACR	19,56±7,37	55,54±69,07	0,138
ESR	18,22±6,59	25,38±16,47	0,233
Creatinine mg/dL, visit 3	1,48±2,07	0,82±0,27	0,263
Fibrinogen mg/dL, visit 3	410,61±85,39	437,31±54,05	0,377
Leukocytes x 10^3/mL visit 3	6,46±1,06	7,60±2,31	0,182
Neutrophils x 10 ^{3}/mL visit 3	3,37±0,64	4,19±1,73	0,192

Table 22. Bivariate analysis of the progression of cognitive impairment.

3.1.3 Progression of cognitive impairment with the inclusion of molecular markers of research: bivariate analysis.

The bivariate analysis is used to determine the association between the research molecules that have been selected with the progression of cognitive impairment.

	No n = 88	Yes n = 13	p
Basal sTWEAK (pg/ml)	5666.1 ±	6956.3 ±2718.6	0.180
sTWEAK at 12 months	2513.2	8649.9 ± 5637.7	0.056
sTWEAK at 24 months	6784.8 ±	8451.1 ±5081.7	0.237
Basal B- A 1-40 (pg/mL)	39.3 ± 26.2	119.4 ±11.1	<0.0001
A 1-40 at 12 months	57.6 ± 41.1	176.3 ± 26.9	<0.0001
A 1-40 at 24 months	40.3 ± 33.9	187.9 ± 33.2	<0.0001
Basal MMP-1 (pg/mL)	3.1 ±1.8	3,1±0,9	0,063
MMP-1 at 12 months	3.6 ± 2.0	3,4±1,6	0,361
MMP-1 at 24 months	3.1 ± 1.8	4,2±1,4	0,256
Basal MMP-3 (pg/mL)	12.4 ± 5.9	11,5±3,4	0,063
MMP-3 at 12 months	12.1 ± 5.4	11,9±3,7	0,361
MMP-3 at 24 months	12.1 ± 5.2	12,4±4,7	0,256
Basal MMP-7 (pg/mL)	5.7 ± 4.1	6,3±2,4	0,315
MMP-7 at 12 months	4.6 ±2.9	6,3±3,3	0,117
MMP-7 at 24 months	5.7 ± 6.6	6,3±3,8	0,783
Basal MMP-9 (pg/mL)	17.1 ± 9.3	15,2±6,3	0,808
MMP-9 at 12 months	15.9 ± 6.7	15,7±8,6	0,935
MMP-9 at 24 months	16.2 ± 8.9	15,4±7,6	0,657
Basal MMP-10 (pg/mL)	1.1 ± 0.8	0,9±0,5	0,721
MMP-10 at 12 months	0.9 ± 0.6	0,9±0,5	0,191
MMP-10 at 24 months	0.6 ± 0.3	0,8±0,3	0,013
Basal MMP-12 (pg/mL)	0.1 ± 0.1	0,1±0,1	0,525
MMP-12 at 12 months	0.2 ± 0.1	0,2±0,1	0,110
MMP-12 at 24 months	0.2 ± 0.1	0,2±0,1	0,013
Basal MMP-13 (pg/mL)	0.7 ± 0.2	0,7±0,1	0,785
MMP-13 at 12 months	0.8 ± 0.1	0,8±0,2	0,987
MMP-13 at 24 months	0.8 ± 0.2	0,8±0,1	0,065
Basal TIMP (ng/mL)	1066.5 406.1	1841,7±1166,4	0,617
TIMP at 12 months	1156.1 326.8	1351,5±333,0	0,409
TIMP at 24 months	772.2 685.9	1281,7±961,4	0,047

Table 23: Bivariate analysis: plasmatic levels of the research molecules in patients with progression cognitive impairment

In patients with progression of cognitive impairment the levels of Aβ 1-40 in basal samples, at 12 and 24 months were significantly higher than in patients in whom no progression was observed. In the case of the other investigated markers (sTWEAK, MMP-1,MMP-3,MMP-7,MMP-9,MMP-10,MMP-12,MMP-13, TIMP) we found no

association **(Figure 13).** We observed no differences in relation to levels of AB 1-40 and profile of cognitive impairment **(Figure 14).**

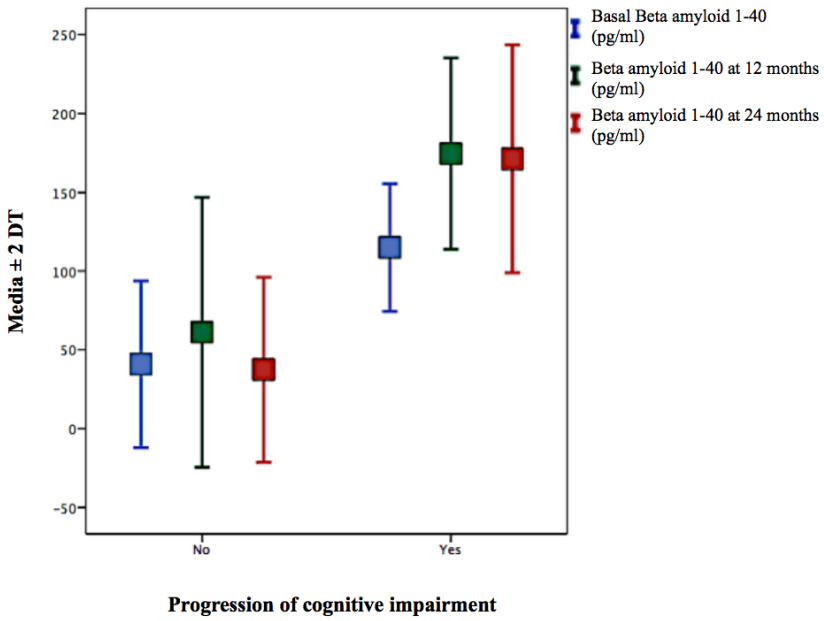


Figure 13: Differences in relation to AB 1-40 levels during the follow-up in patients with progression and without progression of cognitive impairment

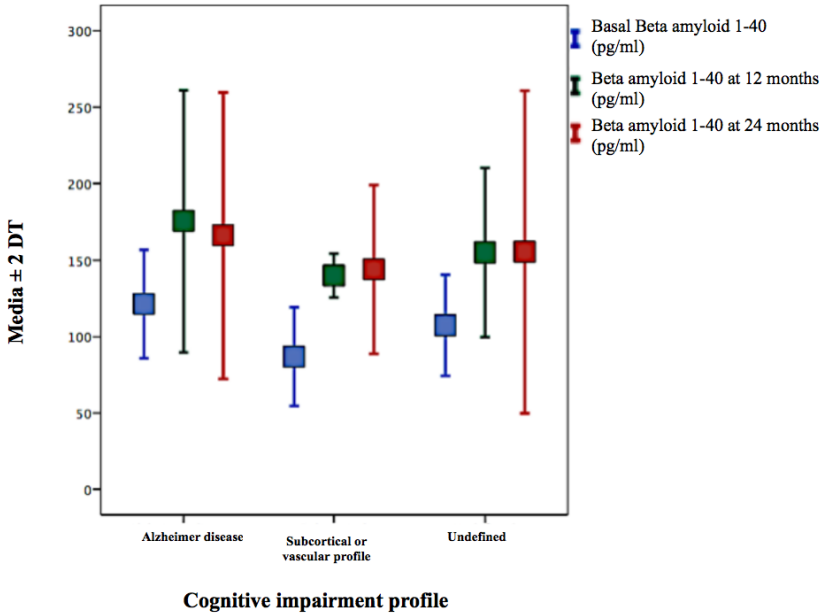


Figure 14: Differences in relation to AB 1-40 levels during the follow-up and profile of cognitive impairment

3.1.4. Progression of cognitive impairment with the inclusion of molecular markers of research: multivariate analysis.

In logistic regression models, we chosen variables with a significance < 0,001 in the bivariate model.

We selected significant molecular markers of research, in these case Aβ 1-40 at 24 months was chosen because the most significant value found in the follow-up was used. The other variables were not associated with progression of cognitive impairment in the bivariate analysis.

In not adjusted and adjusted models the result of OR was 1,02 (CI 1,00-1,03) p 0,005 and 1,02 (CI 1,00-1,03) p 0,016 respectively, so we found that patients with higher levels of Aβ 1-40 develop cognitive

impairment with a discrete higher probability than the group of patients with lower levels **(table 24-25).**

	OR*	CI (95%)	p
AB 1-40 at 24 months	1,02	1,00-1,03	0,005

Table 24: Multivariate analysis: influence of levels of AB 1-40 at 24 months in the progression of cognitive impairment. Crude Odds Ratio produced by a regression model:

	OR**	CI (95%)	p
AB 1-40 at 24 months	1,02	1,00-1,03	0,016

Table 25: Multivariate analysis: influence of levels of AB 1-40 at 24 months in the progression of cognitive impairment. Adjusted Odds Ratio produced by a regression model:

3.2 PROGRESSION OF LEUKOARAIOSIS

3.2.1 Progression of leukoaraiosis: descriptive analysis

With regard to progression of LA we detected 29 patients with increase of grade of LA during evolution. No progression was detected in 72 patients.

Attending to the grade of LA using Fazekas scale at baseline brain MRI, 57 of patients had LA grade I, 25 had LA grade II and 6 had LA grade III. At visit 2 in control MRI brain we detected new appearance of LA in 5 patients (patients who previously did not have leukoaraiosis) and increase of grade in 24 patients. At control brain MRI, 38 of patients had LA grade I, 38 LA grade II and 17 had LA grade III **(Figure 15).**

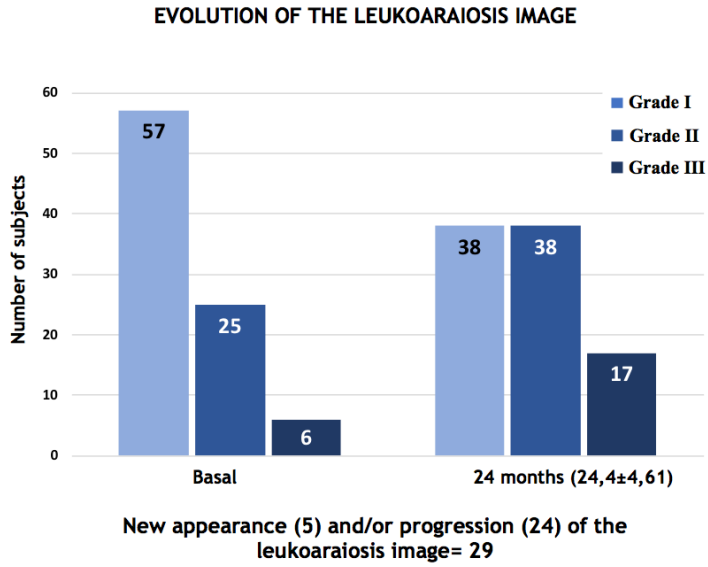


Figure 15: Percentage of LA and grading by Fazekas scale at basal exam and at 24 months of follow-up.

3.2.2 Progression of leukoaraiosis: bivariate analysis

We observed, in group with progression higher percentages of bad clinical control of HT, pathological transcranial IR, new LI and higher levels of ESR and fibrinogen.

93,1% of patients with progression of LA had bad clinical control of HT vs 43,1% of patients with also bad control of HT but no progression ($p< 0,0001$). A trend to present a higher percentage of DLP in the group with progression was observed (86,2% vs 69,4%, $p=0,064$).

62,1% of patients with progression had pathological transcranial IR vs 31,9% of patients with no progression ($p< 0,005$).

Newer LI were detected in group with progression of LA (34,5% vs 6,9%, p= 0,001).

Higher levels of ESR (25,9±10,9 vs 15,9±11,0, p< 0,0001) and fibrinogen (443,2± 74,7 vs 399,1±69,6) were detected in group with progression of LA.

Variable	Progression of leukoaraiosis		
	No n= 72	**Yes n=29**	**p**
Age, years	71,3±5,3	70,2±5,1	0,346
Woman,%	55,6	69,0	0,154
Follow-up time, months	24,4±4,6	26,1±5,3	0,121
BMI, Kg/m^2	30,9±4,9	24,4±3,9	0,108
Bad clinical control of HT, %	43,1	93,1	<0,0001
Bad clinical control of DM2, %	38,1	57,1	0,223
DLP, %	69,4	86,2	0,064
Bad clinical control of DLP, %	30,2	44,0	0,173
Smoking, %	9,7	17,2	0,232
Alcohol consumption, %	11,1	6,9	0,409
ABI pathological, %	11,1	6,9	0,409
New plaques, %	9,7	6,9	0,493
Carotid IP pathological, %	72,2	75,9	0,457
Carotid IR pathological, %	54,2	65,5	0,206
Transcranial IP pathological, %	41,7	58,6	0,093
Transcranial IR pathological, %	31,9	62,1	0,005
Progression of cognitive deterioration, %	50,0	75,0	0,246
New LI,%	6,9	34,5	0,001
New MH, %	13,9	17,2	0,440
ACR	49,5±171,9	64,8±89,8	0,651
ESR	15,9±11,0	25,9±10,9	<0,0001
Creatinine mg/dL, visit 3	0,9±0,8	0,7±0,2	0,270
Fibrinogen mg/dL, visit 3	399,1±69,6	443,2±74,	0,006
Leukocites x 10^3/mL visit 3	6,8±1,7	7,4±1,8	0,125
Neutrophils x 10 ^{3}/mL visit 3	3,6±1,4	4,0±1,3	0,214

Table 26. Bivariate analysis of the progression of cognitive impairment.

3.2.3 Progression of leukoaraiosis with the inclusion of molecular markers of research: bivariate analysis

The bivariate analysis is used to determine the association between the research molecules that have been selected with the progression of LA **(Table 27).**

	No n = 72	**Yes n = 29**	**p**
Basal sTWEAK (pg/ml)	5051,9 ± 2265,9	7862,1±2160,8	<0,0001
sTWEAK at 12 months	5308,6±2453,8	1130,7±4172,5	<0,0001
sTWEAK at 24 months	4557±3286,4	9907,1±4824,3	<0,0001
Basal A 1-40 (pg/mL)	58,4±40,7	61.0 ± 48.1	0.401
A1-40 at 12 months	76,3±56,6	109.1 ±77.4	0.197
A1-40 at 24 months	65,6±68,5	100.3 ± 81.4	0.137
Basal MMP-1 (pg/mL)	31. ± 1.6	3.1 1.6	0.138
MMP-1 at 12 months	3.4 ± 2.1	3.8 1.4	0.297
MMP-1 at 24 months	3.6 ±1.9	2.9 1.3	0.820
Basal MMP-3 (pg/mL)	11.6 ± 4.1	13.2 ± 7.5	0.449
MMP-3 at 12 months	11.7 ±4.6	12.7 ±5.9	0.480
MMP-3 at 24 months	12.4 ±5.1	11.7 ± 5.1	0.619
Basal MMP-7 (pg/mL)	5.8 ± 4.0	5.9 ± 3.3	0.507
MMP-7 at 12 months	4.6 ± 2.8	5.8 ± 3.5	0.535
MMP-7 at 24 months	5.4 ± 5.9	6.6 ± 6.3	0.613
Basal MMP-9 (pg/mL)	16.5 ±9.3	16,7±7,4	0,554
MMP-9 at 12 months	15.5 ± 6.5	16,7±8,5	0,628
MMP-9 at 24 months	16.5 ± 9.1	15,0±7,4	0,641
Basal MMP-10 (pg/mL)	0,8±0,4	1,5±1,0	0,322
MMP-10 at 12 months	0,9±0,7	0,9±0,4	0,463
MMP-10 at 24 months	0,6±0,3	0,7±0,3	0.166
Basal MMP-12 (pg/mL)	0,1±0,1	0,1±0,1	0,578
MMP-12 at 12 months	0,2±0,1	0,2±0,1	0,909
MMP-12 at 24 months	0,2±0,1	0,1±0,1	0,565
Basal MMP-13 (pg/mL)	0,7±0,1	0,7±0,2	0,811
MMP-13 at 12 months	0,8±0,1	0,8±0,2	0,870
MMP-13 at 24 months	0,8±0,2	0,7±0,1	0,799
Basal TIMP (ng/mL)	1122,4±448,2	1536,1±1094,4	0,494
TIMP at 12 months	1254,4±321,3	1105,8±353,5	0,482
TIMP at 24 months	918,3±713,9	862,2±935,6	0,889

Table 27: Bivariate analysis: plasmatic levels of the research molecules in patients with progression of LA

In patients with progression of LA the levels of sTWEAK in basal samples, at 12 and 24 months were significantly higher than in patients in whom no progression was observed **(figure 16).** In the case of the other investigated markers (AB1-40, MMP-1, MMP-3, MMP-7, MMP-9, MMP-10, MMP-12, MMP-13, TIMP) we found no association.

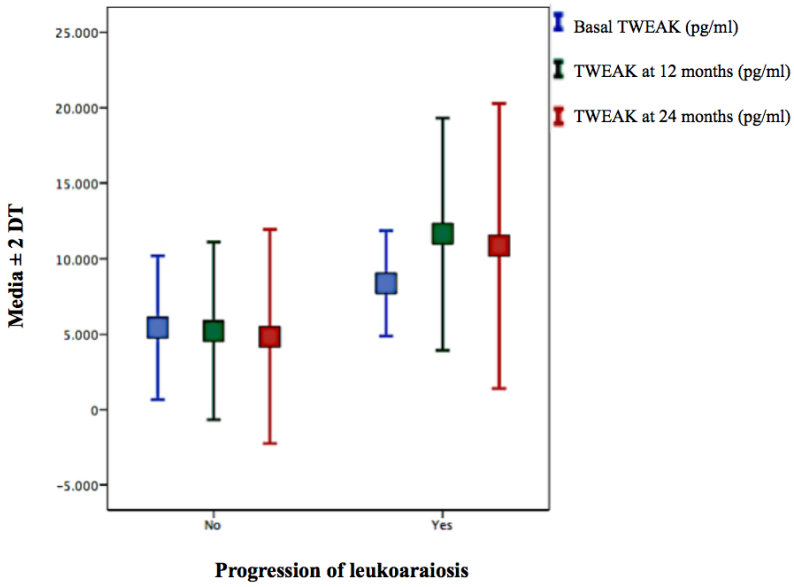


Figure 16: Differences in relation to sTWEAK levels during the follow-up in patients with progression and without progression of LA

3.2.4 Progression of leukoaraiosis multivariate analysis

In not adjusted model the variable that is most associated with progression of LA is bad clinical control of HT (OR 17,8; 95% CI, 3,9-80,8), an association has also been found in patients with pathological transcranial IR (OR 3,5; 95% CI 1,4-8,6), variables that we have also

observed that are associated with the main variable, previously analyzed.

Appearance of new LI is associated with progression of LA (OR 7,1; 95% CI 2,1-23,1).

Higher levels of ESR (OR 1,1; 95% CI 1,0-1,1) and higher levels of fibrinogen during evolution or at visit 3 were observed in patients with progression of LA (OR 1; 95% CI 1,0-1,0) **(table 28).**

In adjusted model the only variable that is associated independently with progression of LA with statistical significance is bad clinical control of HT (OR 7,8; 95% CI 1,5-40,4).

New LI (OR 2,7; 95% CI 0,7-9,8), pathological transcranial IR (OR 2,1; 95% CI 0,7-6,1), higher levels of ESR (OR 1,0; 95% CI 0,9-1,1) and higher levels of fibrinogen during evolution or at visit 3 (OR 1; 95% CI 0,9-1,1) are also associated with progression of leukoaraiosis but without statistical significance **(table 29).**

	OR*	CI (95%)	p
Bad clinical control of HT	17,8	3,9-80,8	<0,0001
Transcranial IR pathological, %	3,5	1,4-8,6	0,006
New lacunar infarcts	7,1	2,1-23,1	0,001
ESR	1,1	1,0-1,1	<0,0001
Fibrinogen visit 3	1	1,0-1,0	0,008

Table 28: Multivariate analysis: influence of variables in the progression of leukoaraiosis. Crude Odds Ratio produced by a regression model.

	OR**	CI (95%)	p
Bad clinical control of HT	7,8	1,5-40,4	0,015
Transcranial IR pathological, %	2,1	0,7-6,1	0,184
New lacunar infarcts	2,7	0,7-9,8	0,139
ESR	1,0	0,9-1,1	0,273
Fibrinogen visit 3	1	0,9-1,1	0,432

Table 29: Multivariate analysis: influence of variables in the progression of leukoaraiosis. Adjusted Odds Ratio produced by a regression model.

3.2.5 Progression of leukoaraiosis with the inclusion of molecular markers of research: multivariate analysis

In logistic regression models, we chosen variables with a significance < 0,001 in the bivariate model.

We selected significant molecular markers of research, in this case sTWEAK at 12 months because the most significant value found in the follow-up was used. The other variables were not associated with progression of LA in the bivariate analysis.

In not adjusted and adjusted models the result of OR was 1,00 (CI 1,00-1,01) p < 0,0001 and 1,00 (CI 1,00-1,01) p <0,007 respectively, so we did not find evidence of association between levels of sTWEAK at 12 months with progression of LA.

	OR*	CI (95%)	p
sTWEAK at 12 months	1,00	1,00-1,01	<0,0001

Table 30: Multivariate analysis: influence of levels of sTWEAK at 12 months in the progression of cognitive impairment. Crude Odds Ratio produced by a regression model.

	OR**	CI (95%)	p
sTWEAK at 12 months	1,00	1,00-1,01	<0,007

Table 31: Multivariate analysis: influence of levels of sTWEAK at 12 months in the progression of cognitive impairment. Adjusted Odds Ratio produced by a regression model

3.3 NEW LACUNAR INFARCTS:

3.3.1 New lacunar infarcts: descriptive analysis

LI were detected in 10 patients on admission, at baseline brain MRI (9,9%). At visit 2 in control brain MRI, 23 patients had LI. New LI were detected in 15 patients (14,9%) **(Figure 17).** Two patients with previous LI, developed new lacunar infarcts.

In patients with new LI, progression of LA is more frequent than in patients without new LI (66,7% vs 22,1%, p= 0,001).

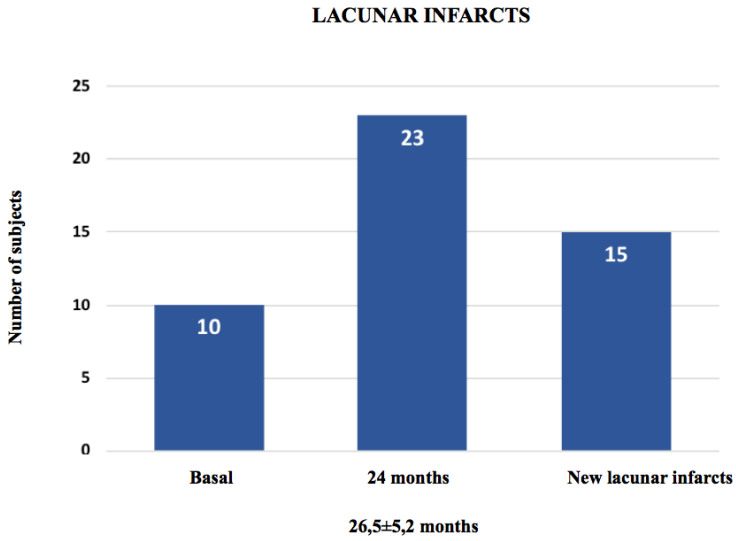


Figure 17: New lacunar infarcts during the follow-up

3.3.2 New lacunar infarcts: bivariate analysis.

We observed, in the group with new LI, higher percentages of bad clinical control of HT , progression of LA , new microhemorrhages and higher levels of erythrocyte sedimentation rate, fibrinogen and leukocytes **(Table 32).**

100% of patients with new LI had bad clinical control of HT vs 50% of patients with also bad control of HT but no progression (p< 0,0001). A trend to present a higher percentage of smoking in the group with progression was observed (26,7% vs 9,3%, p=0,076).

66,7% of patients with new LI had progression of LA vs 22,1% of patients with no new lacunar infarcts (p=0,001) **(Figure 18).**

Newer MH were detected in the group with new LI (46,7% vs 9,3%, p= 0,001).

Higher levels of ESR (27,9±9,8 vs 17,2±11,5 p=0,001), fibrinogen (447,0 ± 82,5 vs 447,0±70,5) and leukocytes (7,9±1,3 vs 6,8±1,8 p= 0,022) were detected in group with new LI.

Variable	New lacunar infarcts		
	No n= 86	Yes n=15	p
Age, years	71,1 ± 9,3	70,4±4,6	0,622
Woman,%	59,3	60,0	0,597
Follow-up time, months	24,8±4,9	25,5±4,8	0,640
BMI, Kg/m^2	30,7±4,8	29,5±3,8	0,378
Bad clinical control of HT, %	50,0	100	<0,0001
Bad clinical control of DM2, %	46,4	42,9	0,602
DLP, %	72,1	86,7	0,195
Bad clinical control of DLP, %	34,4	35,7	0,577
Smoking, %	9,3	26,7	0,076
Alcohol consumption, %	9,3	13,3	0,457
ABI index pathological, %	10,5	6,7	0,543
New plaques, %	9,3	6,7	0,601
Carotid IP pathological, %	70,9	86,7	0,171
Carotid IR pathological, %	57,0	60,0	0,530
Transcranial IP pathological, %	46,5	46,7	0,604
Transcranial IR pathological, %	38,4	53,3	0,210
Progression of cognitive deterioration, %	58,8	60,0	0,684
Progression of LA ,%	22,1	66,7	0,001
New MH, %	9,3	46,7	0,001
ACR	55,2±164,8	46,2±37,4	0,834
ESR	17,2±11,5	27,9±9,8	0,001
Creatinine mg/dL, visit 3	0,9±0,7	0,8±0,3	0,909
Fibrinogen mg/dL, visit 3	405,6±70,5	447,0±82,	0,044
Leukocites x 10^3/mL visit 3	6,8±1,8	7,9±1,3	0,022
Neutrophils x 10^3/mL visit 3	3,6±1,4	4,4±1,0	0,061

Table 32. Bivariate analysis of new lacunar infarcts

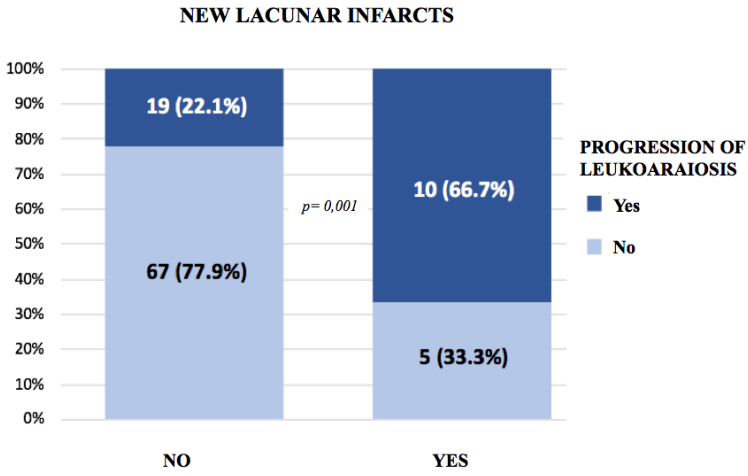


Figure 18: Percentage of patients with new lacunar infarcts and progression of leukoaraiosis

3.3.3 New lacunar infarcts with the inclusion of molecular markers of research: bivariate analysis

The bivariate analysis was used to determine the association between the research molecules that have been selected with the new LI during the evolution **(Table 33).**

In patients with new LI the levels of sTWEAK in basal samples, at 12 and 24 months , MMP-7 at 12 and 24 months, and MMP-9 in basal samples at 12 and 24 months were significantly higher than in patients in whom no LI was observed. In the case of the other investigated biomarkers we found no association.

	New lacunar infarcts		
	No n = 86	**Yes n = 15**	**p**
Basal sTWEAK (pg/ml)	5397,8±2148,8	10124,6±642,3	<0,0001
sTWEAK at 12 months	6082,3±2571,0	15418,5±3997,6	<0,0001
sTWEAK at 24 months	4910,4±2523,6	16351±1811,1	<0,0001
Basal A 1-40 (pg/mL)	60,3±43,5	52,4±39,9	0,403
A1-40 at 12 months	89,6±6,6	71,1±55,2	0,853
A1-40 at 24 months	78,4±73,9	68,9±82,4	0,715
Basal MMP-1 (pg/mL)	2,9±1,6	4,4±0,4	0,001
MMP-1 at 12 months	3,6±1,9	3,3±2,4	0,272
MMP-1 at 24 months	3,3±1,7	4,1±2,4	0,127
Basal MMP-3 (pg/mL)	12,2±5,7	11,7±2,7	0,202
MMP-3 at 12 months	12,2±5,3	10,7±2,8	0,634
MMP-3 at 24 months	12,4±5,2	10,6±2,5	0,127
Basal MMP-7 (pg/mL)	5,0±3,1	11,8±2,5	0,171
MMP-7 at 12 months	4,2±2,3	10,4±1,2	0,014
MMP-7 at 24 months	4,3±4,4	16,6±2,9	0,001
Basal MMP-9 (pg/mL)	14,6±6,9	30,7±4,8	0,001
MMP-9 at 12 months	13,9±4,8	29,6±3,6	0,004
MMP-9 at 24 months	14,4±7,6	27,7±4,5	0,001
Basal MMP-10 (pg/mL)	0,9±0,5	2,1±1,4	0,665
MMP-10 at 12 months	0,9±0,6	1,0±0,4	0,847
MMP-10 at 24 months	0,9±0,3	0,9±0,5	0,004
Basal MMP-12 (pg/mL)	0,1±0,2	0,7±0,1	0,427
MMP-12 at 12 months	0,8±0,2	0,9±0,1	0,501
MMP-12 at 24 months	0,8±0,2	0,8±0,1	0,356
Basal MMP-13 (pg/mL)	0,7±0,2	0,7±0,1	0,427
MMP-13 at 12 months	0,8±0,2	0,9±0,1	0,501
MMP-13 at 24 months	0,8±0,2	0,8±0,1	0,356
Basal TIMP (ng/mL)	1136,8±501,5	2124,7±1536,1	0,552
TIMP at 12 months	1178,8±336,8	1387,8±280,4	0,018
TIMP at 24 months	719,5±385,2	2159,8±1639,5	0,050

Table 33: Bivariate analysis: plasmatic levels of the research molecules in patients with new lacunar infarcts

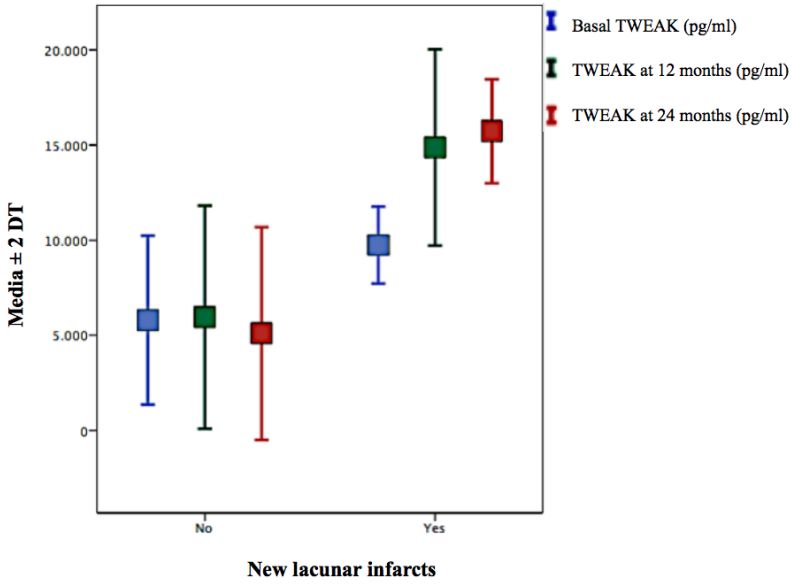


Figure 19: Differences in relation to sTWEAK levels during the follow-up in patients with or without new lacunar infarcts

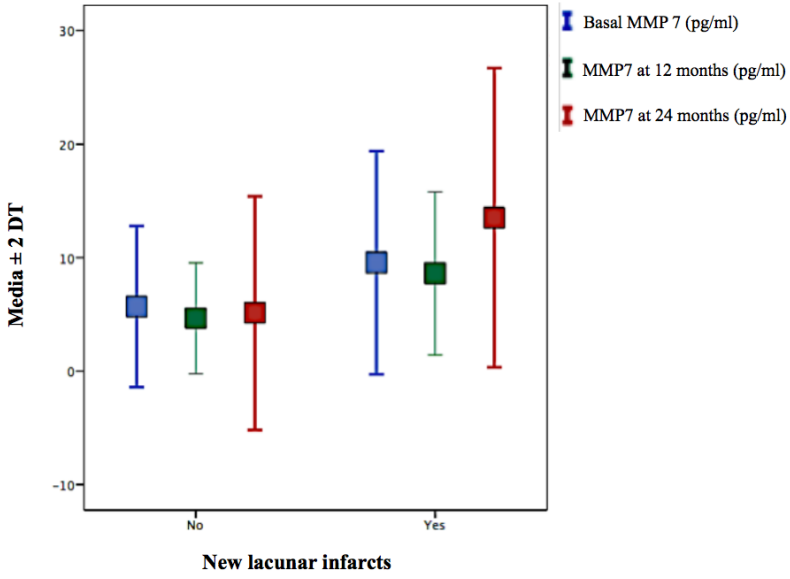


Figure 20: Differences in relation to MMP-7 levels during the follow-up in patients with or without new lacunar infarcts

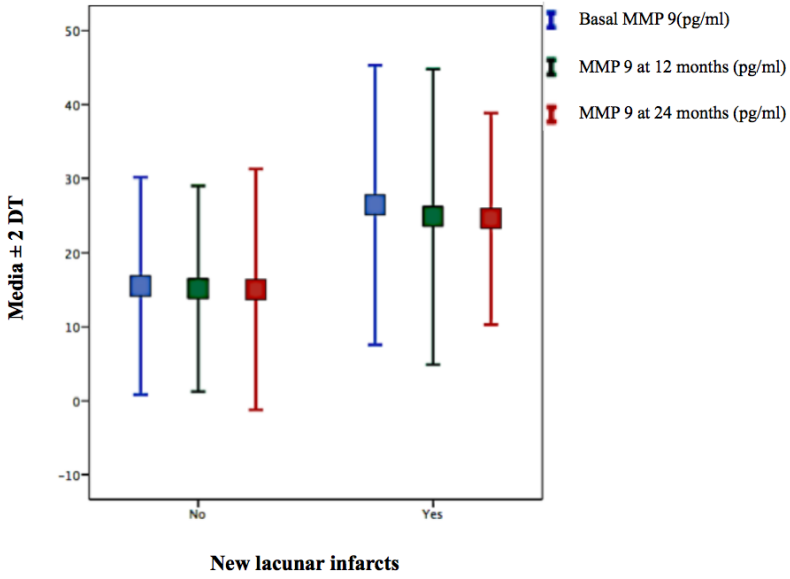


Figure 21: Levels of MMP-9 during the follow-up in patients with or without new lacunar infarcts.

3.3.4 New lacunar infarcts: multivariate analysis.

In not adjusted model the variable that is most associated with new LI is new MH (OR 8,5; 95% CI 2,4-29,7); an association has also been found in patients with progression of LA (OR 7,1; 95% CI, 2,1-23,1).

Higher levels of ESR (OR 1,1; 95% CI 1,0-1,1), higher levels of fibrinogen during evolution or at visit 3 (OR 1; 95% CI 1,0-1,0) and leukocytes at visit 3 (OR 1,4; 95% CI 1,4 1,0-1,9) were observed.

In adjusted model the only variable that is associated independently and with statistical significance with new LI is the appearance of new MH (OR 20,1; 95% CI 3,0-135,1).

Progression of LA (OR 2,9; 95% CI 0,6-13,6) is also associated but without statistically significance.

Higher levels of ESR (OR 1,0; 95% CI 0,9-1,1), higher levels of fibrinogen during evolution or at visit 3 (OR 1; 95% CI 0,9-1,0) and leukocytes (OR 1,3; 95% CI 0,8-2,1) are also associated with appearance of new LI; these variables showed discrete increase of probability to present new LI without statistical significance.

	OR*	CI (95%)	p
Progression of LA	7,1	2,1-23,1	0,001
New MH	8,5	2,4-29,7	0,001
ESR	1,1	1,0-1,1	0,003
Fibrinogen visit 3	1,0	1,0-1,0	0,050
Leukocites visit 3	1,4	1,0-1,9	0,030

Table 34: Multivariate analysis: influence of levels of variables in new lacunar infarcts. Crude Odds Ratio produced by a regression model

	OR**	CI (95%)	p
Progression of LA	2,9	0,6-13,6	0,173
New MH	20,1	3,0-135,1	0,002
ESR	1,0	0,9-1,1	0,627
Fibrinogen visit 3	1,0	0,9-1,0	0,875
Leukocytes visit 3	1,3	0,8-2,1	0,238

Table 35: Multivariate analysis: influence of levels of variables in new lacunar infarcts. Adjusted Odds Ratio produced by a regression model

3.3.5 New lacunar infarcts with the inclusion of molecular markers of research: multivariate analysis

In logistic regression models, we chose variables with a significance < 0,001 in the bivariate model.

We selected significant molecular markers of research; in this case sTWEAK at 24 months , MMP-7 at 24 months and MMP-9 at 24 months were chosen because the most significant value found in the follow-up was used. The other variables were not associated with progression of LA in the bivariate analysis.

In not adjusted and adjusted models the result of OR for sTWEAK was 1,13 (CI 1,06-7,80) p < 0,003 and 1,11 (CI 1,06-5,17) p <0,0076 respectively, so we found evidence of association between levels of sTWEAK at 24 months with new LI with a discrete increase of probability to detect higher levels of sTWEAK in patients with new LI during the evolution in a period of time of two years of follow up **(Table 36-37).**

In not adjusted and adjusted models of MMP-7 at 24 months the result of OR was 1,21 (CI 1,06-1,38) p< 0,005 and 0,93 (CI 0,51-1,68) p < 0,005 respectively, so we have not found association between higher levels of MMP-7 with new LI during the follow-up period **(Table 38-39).**

In the case of not adjusted and adjusted model of MMP-9 at 24 months the result of OR was 1,11 (1,03-1,18) p 0,003 and 1,01 (0,83-1,23) p 0,919 respectively, so no association with new LI was observed in this case either **(Table 40-41).**

	OR*	CI (95%)	p
sTWEAK at 24 months	1,13	1,06-7,80	<0,003

Table 36: Multivariate analysis: influence of levels of sTWEAK at 24 months in new lacunar infarcts. Crude Odds Ratio produced by a regression model

	OR**	CI (95%)	p
sTWEAK at 24 months	1,11	1,06-5,17	<0,006

Table 37: Multivariate analysis: influence of levels of sTWEAK at 24 months in new lacunar infarcts. Adjusted Odds Ratio produced by a regression model

	OR*	CI (95%)	p
MMP-7 at 24 months	1,21	1,06-1,38	0,005

Table 38: Multivariate analysis: influence of levels of MMP-7 at 24 months in new lacunar infarcts. Crude Odds Ratio produced by a regression model

	OR**	CI (95%)	p
MMP-7 at 24 months	0,93	0,51-1,68	0,810

Table 39: Multivariate analysis: influence of levels of MMP-7 at 24 months in new lacunar infarcts. Adjusted Odds Ratio produced by a regression model

	OR*	CI (95%)	p
MMP-9 at 24 months	1,11	1,03-1,18	0,003

Table 40: Multivariate analysis: influence of levels of MMP-9 at 24 months in new lacunar infarcts. Crude Odds Ratio produced by a regression model

	OR**	CI (95%)	p
MMP-9 at 24 months	1,01	0,83-1,23	0,919

Table 41: Multivariate analysis: influence of levels of MMP-9 at 24 months in new lacunar infarcts. Adjusted Odds Ratio produced by a regression model

3.4 NEW MICROHEMORRHAGES

3.4.1 New microhemorrhages: descriptive analysis

MH were detected in 11 patients on admission, at baseline brain MRI, a 10,89% of the sample. At visit 2 in control brain MRI, 20 patients had MH, a 19,8% of the sample. New MH were detected in 15 patients (in 9 patients without previous MH and in 6 patients with previous MH); a 14,85% of the sample **(Figure 22).**

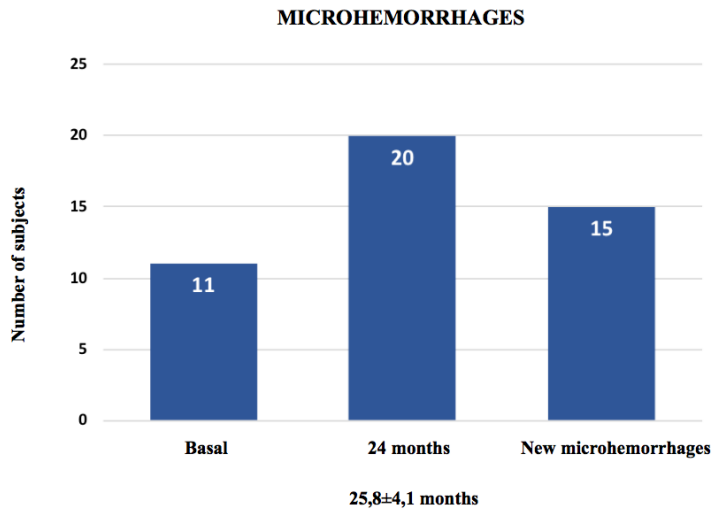


Figure 22: Evolution of percentage of MH from basal to 24 months. New MH during the follow-up.

3.4.2 New microhemorrhages: bivariate analysis

In group with new MH the only variable associated to new MH was new LI. 46,7% of patients with new MH had new LI vs 9,3% of patients with no new MH (p= 0,001).

Variable	New microhemorrhages		
	No n= 86	Yes n=15	P
Age, years	71,0 ± 9,3	71,1±6,1	0,928
Woman,%	60,5	53,3	0,403
Follow-up time, months	24,6±4,9	26,5±4,4	0,183
BMI, Kg/m^2	30,4±4,7	30,7±4,7	0,878
Bad clinical control of HT, %	57,0	60,0	0,530
Bad clinical control of DM2, %	44,8	50,0	0,582
DLP, %	73,3	80,0	0,423
Bad clinical control of DLP, %	35,4	30,8	0,509
Smoking, %	11,6	13,3	0,564
Alcohol consumption, %	10,5	6,7	0,543
ABI pathological, %	11,6	0	0,184
New plaques, %	7,0	20,0	0,129
Carotid PI pathological, %	70,9	86,7	0,171
Carotid RI pathological, %	57,0	60,0	0,530
Transcranial IP pathological, %	46,5	46,7	0,604
Transcranial RI pathological, %	38,4	53,3	0,210
Progression of cognitive deterioration, %	57,9	66,7	0,642
Progression of LA ,%	27,9	33,3	0,440
New LI%	9,3	46,7	0,001
ACR	56,1±164,7	41,5±39,7	0,734
ESR	18,7±11,6	21,7±13,1	0,306
Creatinine mg/dL, visit 3	0,8±0,7	0,9±0,3	0,745
Fibrinogen mg/dL, visit 3	411,5±74,1	413,5±72,5	0,924
Leukocites x 10^3/mL visit 3	6,9±1,8	7,2±1,6	0,633
Neutrophils x 10 ^{3}/mL visit 3	3,7±1,4	3,8±1,4	0,875

Table 42. Bivariate analysis of new microhemorrhages

3.4.3 New microhemorrhages with the inclusion of molecular markers of research: bivariate analysis

The bivariate analysis is used to determine the association

between the research molecules that have been selected with new MH **(Table 43).**

	New MH		
	No n = 86	**Yes n = 15**	**p**
Basal sTWEAK (pg/ml) sTWEAK at 12 months sTWEAK at 24 months	5568,4±2186,2 6302,9±2607,6 5042,3±2609,9	7585,5±3524,3 10845,5±6872,1 11274,3±7092,4	0,084 0,007 0,005
Basal A1-40 (pg/mL) A1-40 at 12 months A1-40 at 24 months	57,7±44,1 92,2±68,0 79,7±77,0	65,4±38,6 68,3±50,4 67,6±62,7	0,971 0,580 0,801
Basal MMP-1 (pg/mL) MMP-1 at 12 months MMP-1 at 24 months	3,1±1,6 3,7±1,9 3,5±1,7	3,2±1,7 2,7±1,8 3,0±2,3	0,587 0,609 0,982
Basal MMP-3 (pg/mL) MMP-3 at 12 months MMP-3 at 24 months	11,8±5,5 11,7±4,6 12,3±5,4	13,6±4,8 13,4±6,6 11,8±3,4	0,014 0,608 0,802
Basal MMP-7 (pg/mL) MMP-7 at 12 months MMP-7 at 24 months	4,2±1,9 3,7±1,7 2,9±1,6	11,9±1,8 9,9±0,9 16,6±2,2	<0,0001 <0,0001 <0,0001
Basal MMP-9 (pg/mL) MMP-9 at 12 months MMP-9 at 24 months	12,5±2,3 12,6±2,3 12,2±3,1	31,9±4,9 28.5±3,1 30,7±5,5	<0,0001 <0,0001 <0,0001
Basal MMP-10 (pg/mL) MMP-10 at 12 months MMP-10 at 24 months	0,9±0,5 0,7±0,3 0,6±0,3	1,6±1,2 1,4±0,9 0,8±0,4	0,592 0,023 0,918
Basal MMP-12 (pg/mL) MMP-12 at 12 months MMP-12 at 24 months	0,1±0,1 0,2±0,1 0,2±0,1	0,1±0,1 0,2±0,1 0,2±0,1	0,874 0,742 0,800
Basal MMP-13 (pg/mL) MMP-13 at 12 months MMP-13 at 24 months	0,7±0,2 0,8±0,1 0,8±0,2	0,7±0,1 0,8±0,2 0,8±0,1	0,788 0,881 0,988
Basal TIMP (ng/mL) TIMP at 12 months TIMP at 24 months	1144,6±486,3 1176,7±341,5 699,4±366,8	1700,0±1306,9 1312,0±303,8 1660,4±1388,5	0,611 0,250 0,005

Table 43. Bivariate analysis: plasmatic levels of the research molecules in patients with new MH

In patients with new MH the levels of sTWEAK in basal samples, at 12 and 24 months , MMP-7 at 12 and 24 months, and MMP-9 in basal samples at 12 and 24 months were significantly higher than in patients in whom no new MH was observed **(Figure 23, 24, 25).** In the case of the other investigated biomarkers (MMP-1,MMP-3, MMP-10, MMP-12,MMP-13, TIMP and AB1-40), we found no association.

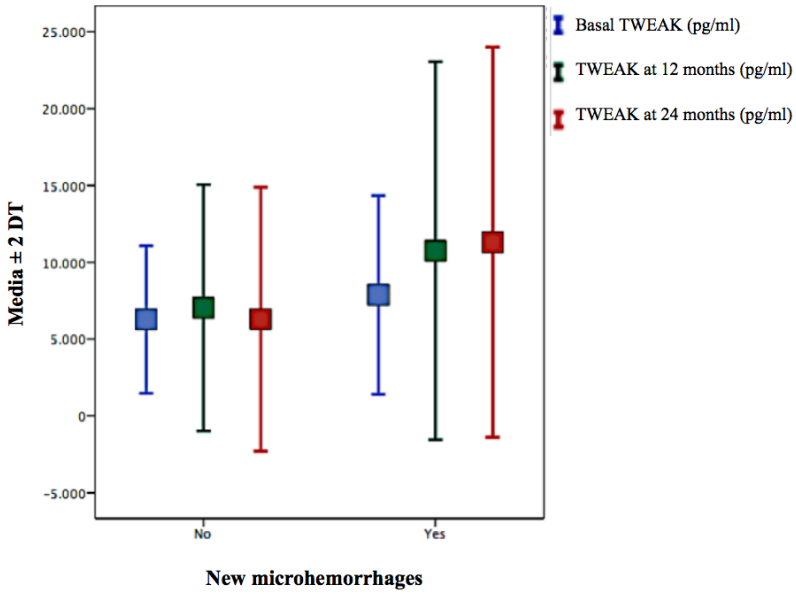


Figure 23: Differences in relation to levels of sTWEAK during the follow-up in patients with new MH or not

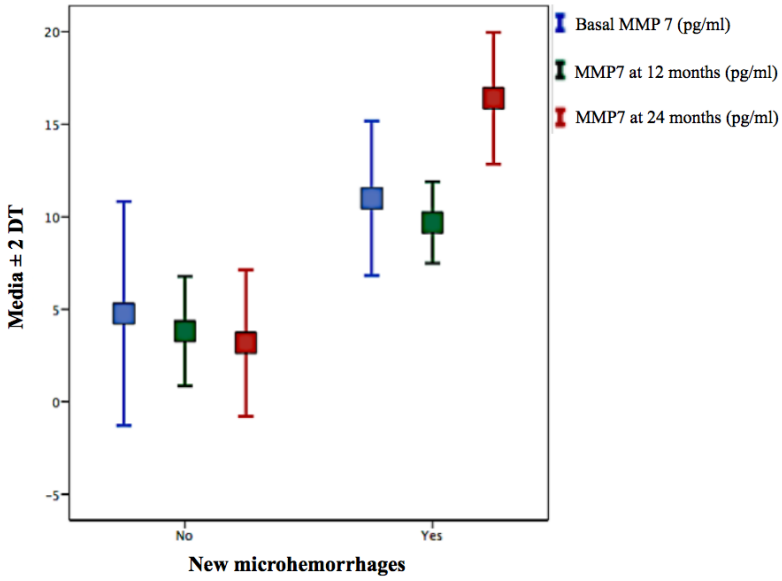


Figure 24: Differences in relation to levels of MMP-7 during the follow-up in patients with new MH or not

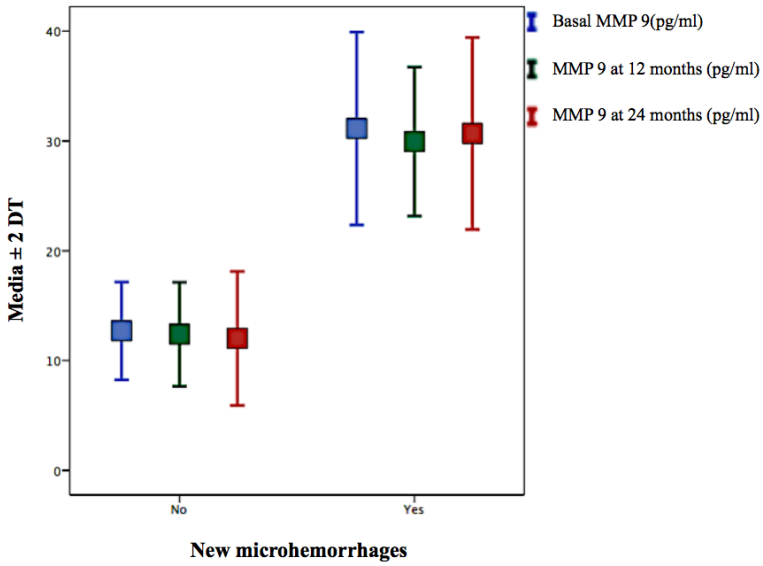


Figure 25: Differences in relation to levels of MMP-9 during the follow-up in patients with new MH or not

3.4.4 New microhemorrhages: multivariate analysis

In not adjusted model the variable that is most associated with new MH is new LI (OR 8,5; 95% CI 2,4-29,7).

No variable was associated independently with new MH **(Table 44-45).**

	OR*	CI (95%)	p
New LI	8,5	2,4-29,7	0,001

Table 44: Multivariate analysis: influence of new lacunar infarcts in the apparition of new MH. Crude Odds Ratio produced by a regression model

	OR**	CI (95%)	p
New LI			

Table 45: Multivariate analysis: influence of new lacunar infarcts in the apparition of new MH. Adjusted Odds Ratio produced by a regression model

In not adjusted model the variable that is most associated with new MH is new LI (OR 8,5; 95% CI 2,4-29,7) **(Table 44)**

No variable was associated independently with new MH **(Table 45).**

3.4.5 New microhemorrhages with the inclusion of molecular markers of research: multivariate analysis

In logistic regression models, we chose variables that in the bivariate model had a significance < 0,001.

We selected significant molecular markers of research, in this case sTWEAK at 24 months, MMP-7 at 24 months and MMP-9 at 24 months were chosen because the most significant value found in the follow-up was used. The other variables were not associated with progression of MH in the bivariate analysis.

In not adjusted and adjusted models the result of OR for sTWEAK was 1,00 (CI 1,06-7,80) p < 0,014 and 1,00 (CI 0,86-1,12) p <0,188 respectively, so we found no evidence of association between levels of sTWEAK at 24 months with new MH in a period of time of two years of follow-up **(Table 46-47).**

In not adjusted and adjusted models of MMP-7 at 24 months the result of OR was 1,87 (CI 1,02-2,15) p< 0,007 and 1,85 (CI 1,03-2,32) p < 0,001 respectively, so we have found a positive association between higher levels of MMP7 with new MH during the follow-up period of two years **(Table 48-49).**

In the case of not adjusted and adjusted model of MMP-9 at 24 months the result of OR was 1,18 (1,01-3,16) p 0,007 and 1,21 (1,00-3,12) p 0,919 respectively, so a positive association between higher

levels of MMP-9 with new MH was also observed in this case **(Table 50-51).**

Higher levels of MMP-7 and MMP-9 are associated independently and with statistical significant with an increase of probability of new MH during the follow-up of two years. Taking into account the values of OR the most associated of the two variables is MMP-7.

	OR*	CI (95%)	p
sTWEAK at 24 months	1,00	1,00-1,00	0,014

Table 46: Multivariate analysis: influence of levels of sTWEAK at 24 months in new MH. Crude Odds Ratio produced by a regression model

	OR**	CI (95%)	p
sTWEAK at 24 months	1,00	0,86-1,12	0,188

Table 47: Multivariate analysis: influence of levels of sTWEAK at 24 months in new MH. Adjusted Odds Ratio produced by a regression model

	OR*	CI (95%)	p
MMP7 at 24 months	1,87	1,02-2,15	<0,007

Table 48: Multivariate analysis: influence of levels of MMP-7 at 24 months in new MH. Crude Odds Ratio produced by a regression model

	OR**	CI (95%)	p
MMP7 at 24 months	**1,85**	**1,03-2,32**	**<0,0001**

Table 49: Multivariate analysis: influence of levels of MMP-7 at 24 months in new MH. Adjusted Odds Ratio produced by a regression model

	OR*	CI (95%)	p
MMP9 at 24 months	1,18	1,01-3,16	0,007

Table 50: Multivariate analysis: influence of levels of MMP-9 at 24 months in new MH. Crude Odds Ratio produced by a regression model

	OR**	CI (95%)	p
MMP9 at 24 months	1,21	1,00-3,12	0,019

Table 51: Multivariate analysis: influence of levels of MMP-9 at 24 months in new MH. Crude Odds Ratio produced by a regression model

4. EFFECT OF HYPERTENSION IN MARKERS OF SVD

In our study we have obtained a positive and statistically significant relationship between bad clinical control of hypertension and the progression of any SVD phenotype OR 4,73 CI (95%) (1,18-18,91) p = 0,028 and progression of LA OR 7,8 (1,5-40,4) p = 0,015. **(figure 26).**

A positive association has been observed in the relationship between the non-dipper pattern and the white coat effect 44,2% vs 55,6% in the group with progression and 42,4 vs 57,6% in the group with progression respectively. No multivariate analysis has been performed for theses variables. The association of these variables with secondary variables of the study has not been analysed either.

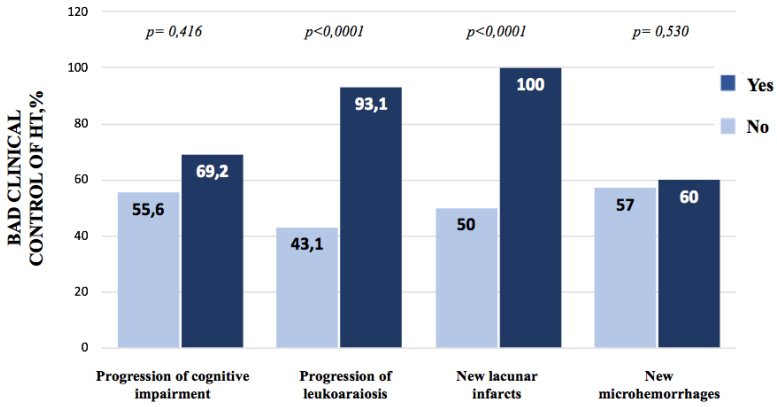


Figure 26: Relationship between bad clinical control of HT and progression of cognitive impairment, leukoaraiosis, new lacunar infarcts and new microhemorrhages

Bad control of hypertension was associated also with the risk of progression of severe LA vs mild with a OR of 7,8 (CI 95% 1,5-40,4; p 0,015) however, no significant association has been established with the other secondary study variables.

5. RELATION BETWEEN ULTRASONOGRAPHY MARKERS AND PROGRESSION OF SVD

Ultrasonographic markers of high pulsatility and resistance in our study were not independently associated after being adjusted for other variables. However were higher in the all phenotypes of SVD progression, specially in the case of progression of LA (62,1% vs 31,9%) p = 0,005 **(figure 27).**

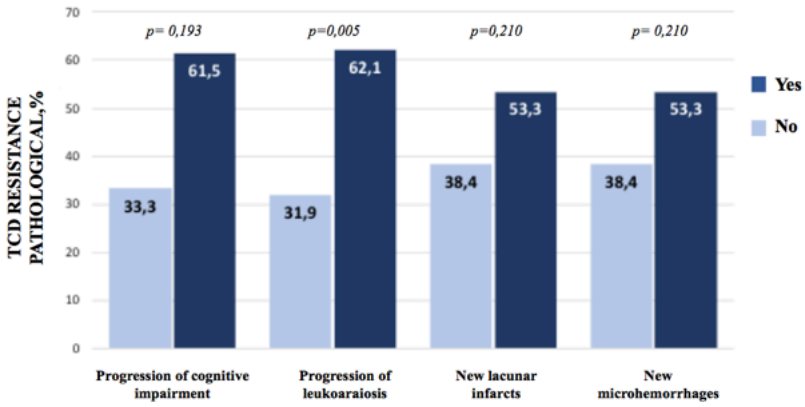


Figure 27: Relationship between transcranial pathological RI and progression of cognitive impairment, leukoaraiosis, new lacunar infarcts and new microhemorrhages

DISCUSSION

The main objective of the study is to identify a clinical risk profile for the development of phenotypes of SVD. A lot of previous studies have analysed the association of vascular risk factors with the presence of markers of SVD but there are only a few longitudinal studies and there are no studies investigating biomarkers related to the progression of the disease. Our objective is to detect the risk factors and investigate if the degree of control of these factors could influence the progression of leukoaraiosis, the appearance of new lacunar infarcts, new microhemorrhages or the worsening of cognitive impairment. For this purpose, we designed a longitudinal study and used a cohort sample with a high probability of presenting SVD.

We have obtained an important correlation, as we will see, with the studies of progression of the SVD published to date, for this reason, we consider that the data obtained of this work have scientific validity. It is necessary to point out that we have found differences in relation to studies in which patients with symptomatic SVD were followed, such as those who presented a non-silent lacunar stroke.

1. PREVALENCE OF RADIOLOGICAL MARKERS OF SVD

Our sample contains patients with a high risk of presenting SVD but who are asymptomatic from neurovascular point of view.

In the initial assessment 87,1% of the sample had radiological or clinical markers. Only a 12,9% of patients had none of any phenotype of SVD. The most frequent finding was the presence of leukoaraiosis in 69% of the patients; other radiological findings were lacunar infarcts in 9,9%, and microhemorrhages in 10,9%.

These results have also been observed in other epidemiological studies of patients with similar baseline characteristics as we will see below.

1.1 LEUKOARAIOSIS

White matter hyperintensities (WMH) or leukoaraiosis (LA) were detected at basal MRI in 87,1% of the sample. 56% had LA grade I or mild, 24,8% had LA grade II or moderate and 5,9% had leukoaraiosis grade III or severe.

In the Rotterdam Scan Study (475), the prevalence of lesions of cerebral white matter in asymptomatic patients is analysed, and it occurs in approximately 87% of patients between 60-70 years. This population is similar to our study(with average age of 71 years).

In the Austrian Stroke Prevention Study (29), at baseline, 64,5% had leukoaraiosis, 52% grade I, 9,2% grade II and 3,3% grade III, but the median age was 60 ± 6,1 years and the prevalence of hypertension was lower than in our study.

1.2 LACUNAR INFARCTS

Lacunar infarcts (LI) were detected at basal MRI in 9,9% of the sample in our study.

Studies of prevalence of MRI *silent brain infarcts* in general population showed results around 8-28% (476).

Prevalence of silent brain infarcts in Rotterdam Scan Study at basal MRI was 20% with elderly sample without dementia and mean age of 72 years (477); in Framingham Heart Study the prevalence was 12% with general population without stroke or dementia and mean age 62 years (478); in Helsinki Aging Brain Study (479) the prevalence was 16% in elderly sample mean age 72 years without neurological disease, in Memory and Morbidity in Augsburg Elderly study the prevalence was 13% in elderly sample with mean age of 72 years (480).

We obtained a lower prevalence than most of the previously described studies since we only considered silent lacunar infarcts and the others considered silent infarcts (not only lacunar infarcts).

When we specifically search *silent lacunar strokes* in studies of research the prevalence is not so variable.

In 786 patients with first ever ischemic stroke and mean age of 59,5%, the prevalence was 10,3% for a single silent lacune and 11,1 %

for multiple silent lacune (481). In 477 participants of PATH Through Life Study aged 60-64 years without previous history of stroke or dementia the prevalence of silent lacunar infarct was 7,8% (482).

1.3 MICROHAEMORRHAGES

In our study we obtained a higher prevalence of microhemorraghes (MH) than in other studies that analysed this prevalence in healthy people. We obtained a 10,89% at basal MRI, a 19, 8% at the end of follow-up period and a 14,85% if we consider new MH during the follow-up. These differences may be due to the fact that our sample is older and there is a greater proportion of hypertension. We found more located in in lobar regions (60%) than in basal ganglia and brainstem (40%) during evolution.

Prevalence of MH was studied in *systematic review* by Cordonnier in 2007 (33). Results are based on studies realized in people without neurological diseases and asymptomatic recruited from Framingham and Offspring cohorts and in people with cerebrovascular disease. Prevalence in healthy people in this review was a 5%. However, in patients with previous ischemic stroke was 33,55% and prevalence among people with any cerebrovascular disease (including previous ICH) was higher, in this group was 45,4%.

Prevalence of MH was 6,4% in the study designed by Roob et al in 1999 with 280 patients. This percentage was lower than in our sample, but the average age was lower (60 years) and only 32% of the patients included were hypertensive. Hypertension was related to presence of MH ($p<0,001$). In this study LA in 67% of patients and lacunes or LI in 8% were detected concomitantly (483).

Other studies showed the same. In studies where the average age is lower, the prevalence is also inferior; for example, in the study of Tsushima et al in 2002 in 450 healthy Japanese of 53, the prevalence of MH of 3,1%. These lesions were also related to hypertension ($p< 0,0001$) in these study (484).

In the study of Horita et al also in Japanese population, MH was demonstrated in 7,7% of healthy people, with mean age of 56,4 years.

Hypertension (OR 7,0; 95% CI 1,4-34,7) and age > or = 65 years old (OR: 5,9; 95% CI 1,4-25,9; p= 0,02) were associated independently with MH (485).

In Framingham cohort (486) with 472 patients the prevalence of MH was 4,7%, mean age was 64,4, but prevalence in subjects older than 75 years the prevalence was 12,6%. In this study they found a positive association between MH and hypertension but in adjusted model the relationship was no significant. Like our sample, they found that the predominant location was in cortex and subcortical areas (73%) over basal ganglia and brainstem (27%), probably related to progression of CAA.

2. VASCULAR RISK FACTORS IN SMALL VESSEL DISEASE

2.1 HYPERTENSION

2.1.1. Prevalence of hypertension:

Hypertension is considered the main risk factors for the development of this disease (487). For this reason, in this study the effect of blood pressure is exhaustively investigated, taking into account the degree of clinical control (in doctor´s office), ambulatory by 24hours monitoring (AMBP) and pattern in bedtime or nocturnal hypertension, the presence of white coat effect and resistant hypertension. In this study we analysed the prevalence of all these characteristics of hypertensive patients.

Our sample has an average age of 70 years and there is a similar proportion of men and women. 99% of the patients were hypertensive. Clinical bad-controlled hypertension was found in 57,4%, but this percentage dropped to 20,8% when we analyse the results of the AMBP.

In Spain, according to results of Diabet.es study dated in 2016, the prevalence of hypertension in general population older than 60 years is

75,4% and in people age > 75 years is 88,7% (488). In this study only 30% had good clinical control of BP (489).

In the systematic review and meta-analysis of 76 epidemiological studies on 341632 patients conducted in Spain, the prevalence of uncontrolled hypertension was 67%, rising up to 87,6% when the most restricted definition (≥130/80-85 mmHg) was used (490).

We obtained better control of blood pressure in our sample. One reason could be the fact that patients visited their primary care physician every 6 months to check the degree of control of their risk factors.

It is important the use of ABMP also to defined control of HT because most of the patients experience a reaction of alert (anxiety) during the arterial pressure measurement in doctor office that can be very important in some cases. This is known as white coat effect. White coat effect was detected in 33,3% of the sample. Similar percentage of white effect (37,5%) was detected in study directed by de la Sierra et al in 8295 patients with resistant hypertension (491).

23,8% of the sample has 3 or more antihypertensive drugs (including at least one diuretic). Resistant hypertension was detected in the 11,9% of the sample. Similar prevalence of resistant hypertension, 12,8% was reported in United States between 2003-2008 in adults (492).

44,7% of subjects had a diminished nocturnal blood pressure decline pattern (non-dipper and reverse dipper pattern). Similar results of dipping pattern, 43% non-dippers (including reverse-dippers) were found in 575 hypertensive subjects older than 50 years (493).

2.1.2 Effect of hypertension in markers of SVD:

In our study we have obtained a positive and statistically significant relationship between bad clinical control of hypertension and the progression of any SVD phenotype, a positive association has been observed also in the relationship between the non-dipper pattern and the white coat effect.

In other studies association between no dipper pattern and silent cerebral infarcts was found but the sample was bigger (493). However,

in the case of LA, the relationship between the alteration of the nocturnal pattern of decrease in blood pressure and the degree of LA has not been demonstrated in other studies (494).

Most studies analyse the variable *medical history of hypertension* and not the control of hypertension. This is the case of LADIS study, that analysed whether the presence of hypertension was associated with the increase of severity of SVD. In LADIS study with 639 subjects that were followed for 3 years, with 395 patients with previous history of hypertension and 53,5% of patients were treated with antihypertensive at time of inclusion. In this study history of hypertension was not associated with WMH progression OR 1,2 (0,6-2,1) however, the analysis of history of hypertension was associated with an increase of severity of LA in the subgroup of patients without stroke. History of hypertension was a baseline predictor of new lacunes during the follow-up with OR 2 (1,1-3,9) p <0,05 (495). In LADIS study the presence of systolic hypertension was predictor of development of new lacunes OR 1,3 (1,1-1,6) p<0,001 but diastolic hypertension was not predictor.

Recently the effect of antihypertensive medication on SVD was reviewed in meta-analysis published in 2018 that analysed study characteristics of ACCORD-MIND (intervention was systolic blood pressure < 120 mmHg), PRoFESS (intervention was the treatment with Telmisartan), PROGRESS (intervention was the treatment with Perindopril and Indapamide) and SCOPE (intervention was treatment with Candesartan). The intervention with antihypertensive treatment was effective in slowing the progression of WMH with a standardized mean difference -0,19 (95% CI, -0,32 to -0,06) (496).

Bad control of hypertension unlike the other markers analysed in our study and unlike another studies reviewed previously was not related to the appearance of new MH; one reason for the lack of relationship could be the minor proportion of MH located in basal ganglia and brainstem in the second MRI. Lesions located in basal ganglia and brainstem are likely to be related to hypertension.

2.2 DIABETES

2.2.1 Prevalence of diabetes mellitus

At the time of inclusion, 36.6% of the patients had diabetes and during the evolution 45.7% were poorly controlled. In a registry made in Valencia, Spain, with more than 5000 patients with diabetes, a 57% of them had uncontrolled diabetes defined by HbA1c levels (490).

2.2.2 Effect of diabetes in markers of SVD

In the bivariate analysis, no association was found between the presence of poor control of diabetes with any radiological marker of progression of cerebral microangiopathy or with the progression of cognitive deterioration. Type 2 diabetes mellitus (DM2) is an established risk factor for ischemic stroke and cognitive decline but the relationship with SVD is inconsistent (497)(498).

First, we will comment the studies with positive association. According to previous studies, it is known that diabetes increases the prevalence of lacunar stroke compared to other subtypes of strokes and it has also been observed that the risk of presenting lacunar infarctions is two times more frequent in patients with diabetes than in patients who do not have it (499), (500).

Reviewing the studies in this regard it seems that the association between diabetes with cerebral microangiopathy is clear in the brainstem lacunar infarcts, but is not unified in the case of presence of white matter hyperintensities and silent brain infarcts (501).

In the case of SVD, diabetes is involved in the process of lipohyalinosis, and lacunar infarcts are more frequent, but specially in posterior circulation by branch atheromatous disease caused by microatheroma at the orifice of the penetrating artery (502).

Highlight the cross sectional studies with conflicting results such as the published by Manshot et al in 2006 where relationship between the presence of diabetes and neuroimaging markers of SVD was found. Diabetes in this study is associated with the presence of deep WMH ($p=0,02$) and specially with cortical WMH ($p< 0,001$). Also in this

study they found association with impairment of cognitive function and atrophy (503).

Similar results were published by Harten in 2007 in a study with patients with DM2 and hypertension. They found that DM2 was an independent risk factor for deep white matter hyperintensities (504).

And even, Jongen et al in 2007 found similar results in an almost 100 patients with DM2. Results were that DM2 was related to higher volume of white matter hyperintensities and atrophy, suggesting relationship with mixed neurodegeneration in the brain (505).

However, in studies realized also in DM 2 patients and controls such as those made by Umemura et al in 2008, De Bresser et al, van Elderen et al in 2010 (501), it was not found significant relationship between diabetes and leukoaraiosis or silent lacunar infarcts.

Few studies have specifically analysed the relationship between glycaemic control and progression of SVD. The ACCORD MIND clinical trial (2011) with 2977 participants that the intensive treatment to type 2 diabetic patients with the objective of HbA1c less than 6% did not improve the outcomes of the study (cognitive impairment, total brain volume and white matter lesions volume), even there was more WMH volume in the intensive group compare to standard (506).

A clear association between diabetes and cognitive impairment of Alzheimer´s type has been observed in relation to non-diabetic patients in previous studies (507),(508).

In relation with DM2 and cognitive impairment in our study only a 15,9% of the patients with cognitive impairment had a pattern of AD. The lack of association between poor control of diabetes with cognitive impairment may be due to a small sample size and that we did not evaluate the degree of brain atrophy to identify more clearly the cases of cognitive deterioration in which an AD may underlay.

2.3 DYSLIPEDEMIA

2.3.1 Prevalence of dyslipidemia

74,3% of the sample had dyslipidemia at inclusion of which 65,4% had good control and 34,6% bad control. The prevalence of persistent

lipid abnormalities in patients receiving statins is higher than in our sample; in European and Canadian population it was 48,2% in 2012 registry study with 22.000 patients (509).

2.3.2 Effect of dyslipidemia in markers of SVD

Compared with studies that analyzed the possible association of dyslipidemia with SVD such as 3C Dijon Study and EVA Study with previous history of hypercholesterolemia of 56,7% and 66,6% respectively, in our study we observed a higher prevalence of dyslipidemia. The main age in these studies were 73 and 69 years, the presence of hypertension 76,9% and 54,7% and the presence of diabetes was 8,6% and 1,3% respectively and smoking was present in the 3C Dijon Study in 39% of the sample and in 10% of the sample in the EVA study.

These differences, mainly in the presence of diabetes and a high percentage of smokers are important with respect to our data, since our patients had a high load of vascular risk factors (except for smoking).

In our study we didn´t find association between the presence of previous history of dyslipidemia (or statin uptake) and progression of any marker of small vessel disease or in the secondary variables (cognitive deterioration, leukoaraiosis progression, new lacunar infarcts and new microhaemorrhages). These results are concordant with the previously mentioned studies (3C Dijon Study and EVA) that only found association between neuroimaging markers of SVD and hypertriglyceridemia but didn't find association with other lipid fractions. In our study levels of triglycerides were not taken into account for the statistical analysis (510).

Another previous studies such as LADIS and the Cardiovascular Health Study did not find an association between LDL cholesterol and neuroimaging markers (398), (511).

2.4 OTHER RISK FACTORS

2.4.1 Obesity according to body mass index

In our sample regarding classification of BMI: 7,9% of the sample were no obese, the 45,5% were overweight, the 28,7% were obesity type I, 11,9% obesity type II and 5,9 were obesity type III. In the group of no progression we observed higher levels of BMI.

In bivariate and multivariate analysis, patients with higher BMI had less progression of SVD and an inverse association was observed (OR 0,84; 95% CI 0,73-0,97), so surprisingly, higher BMI remains as a protective factor to progression of SVD.

The obesity paradox was replicated as protective condition in terms of morbidity and mortality in several studies about cardiovascular diseases, cancer and systemic diseases as rheumatoid arthritis (512). However, using BMI to classify individuals as overweight or obese has major limitations, because it doesn't distinguish fat from muscular mass; also BMI doesn't offer information about distribution or fat features. Visceral fat is in the fact the main predictor of new-onset diabetes and is associated with cardiovascular mortality in Framingham cohort (513–515).

In relation to the molecular mechanism of the obesity paradox there are evidences about the protective effect of subcutaneous fat. This fat does not have adipose tissue inflammation with systemic metabolic consequences, however in this fat there are angiogenesis potential, low extracellular matrix fibrosis and low macrophages infiltration/activation among other capabilities (516).

In relation with our study waist circumference was not associated with less or more progression of SVD.

Elderly people, such as in our sample, generally possess much lower BMI and more comorbidities for cardiovascular disease and diseases that induce unintentional weight loss, creating a bias for obesity paradox (517). This relationship could be due to confounding factors for obesity paradox such as lower resistance to cachexia in patients with SVD progression, that could imply progression of

cognitive deterioration. Weigh loss is frequent in progression of mild cognitive impairment to dementia (518).

3. COGNITIVE IMPAIRMENT

Cognitive impairment was found in 21,8% at basal evaluation but only 2,9% of the sample had cognitive impairment of subcortical profile. In 13 patients (59,1%) we observed progression of patients with cognitive impairment at basal examination.

SPS3 trial obtained, over a median follow-up of 3 years, that the incidence of mild cognitive impairment in patients without cognitive impairment was around 9,5-10% per year (519). In our study, 6,9% of the sample presented mild cognitive impairment during the follow-up though it was not present at basal assessment. Probably we obtained less incidence because all of the patients in SPS3 had had a recent symptomatic lacunar infarct. There is an important evidence that cognitive impairment appears to be frequent after lacunar infarcts despite their size (520).

We obtained that high serum level of Aβ 1-40 was associated with increased risk of progression of cognitive impairment at follow up (OR 1,02; CI 95% 1,00-1,03). Aβ protein in CAA is predominately composed of Aβ 1-40 and in smaller proportion of Aβ 1-42; the increase of Aβ 1-40 in plasma could be due to an increase of the cerebral amyloid soluble burden (521).

We didn´t find an association between vascular risk factors, neuroimaging markers of SVD, ultrasonography markers of arterial stiffness and molecular markers of inflammation and cognitive impairment probably in relation to the small sample size of patients with cognitive impairment and because we only found a subcortical profile in 2,9% of the sample and AD or indeterminate profile in the rest (22,8%).

We did not find either an association between arterial stiffness expressed by supra-aortic or transcranial pulsatility and pathological resistance. This is in accordance with the published scientific literature

about the existence of a weak association between these two factors (522). It could be due to the fact that the mechanisms other than microvascular disease, play a role in the pathobiology of cognitive impairment such us neurodegenerative pathology.

4. PLASMA AMYLOID B LEVELS

We investigated plasma levels of Aβ 1-40 using ELISA, and obtained that higher plasmatic levels of Aβ1-40 are associated with progression of any phenotype of the disease and with progression of cognitive impairment. OR 1,05; CI 95% (1,00-1,10) and OR 1,02 CI 95% (1,00-1,03) respectively.

There is evidence that, in the cases of amyloid angiopathy, plasma levels of Aβ 1-40 and 1-42 are elevated and decreased in CSF, mainly Aβ1-40. Aβ1-40 is deposited in vessels in CAA (214). There are also studies in Alzheimer's Disease with contradictory results, such as Framingham Study participants with lower levels in plasma associated with risk of incident dementia (523); it was attributed to the fact that probably the plasma concentrations of Aβ change in the preclinical phases of the illness.

In the Swedish bio-FINDER study they measured levels of Aβ1-40 and Aβ1-42 in plasma and CSF using the Simoa ultrasensitive digital ELISA in normal subjects and in patients with subjective cognitive decline, mild cognitive impairment, and in AD and correlated this levels with Aβ plaque burden using amyloid PET imaging. Plasma levels of Aβ42 and Aβ40 were reduced in AD dementia compared with control and preclinical stages. However, during the preclinical or prodromal AD stages (i.e. in amyloid positive controls, subjective and mild cognitive impairment) plasma concentration of Aβ42 was just moderately decreased whereas Aβ40 levels were unchanged; additionally, higher plasma levels of Aβ were associated with WMH, cerebral MH, hypertension and diabetes (524). The lower level of the ratio Aβ42/ Aβ 40 is associated with cognitive impairment or AD onset (525,526).

With regard to small vessel disease, higher levels of Aβ 1-40 and Aβ 1-42 are associated with the progression of WML and the presence of LI in patients with cognitive impairment of vascular cause and also in patients with AD. Additionally, studies previously published linked high levels of Aβ1-40 with progression of cognitive impairment in cognitively normal adults. In the Rotterdam Study a prospective cohort study with elderly people without dementia, obtained higher levels of Aβ1-38, Aβ1-40, Aβ1-42 and Aβ1-40/Aβ1-42 that were associated with higher volume of WMH, silent LI, and with worse results in cognitive test specifically in memory (527).

5. PROGRESSION OF SVD

5.1 PROGRESSION OF ANY PHENOTYPE OF SMALL VESSEL DISEASE

Progression of any phenotype of SVD was detected in 42,6% of patients during the follow up. The most frequent phenotype that progressed was LA.

5.2 PROGRESSION OF LEUKOARAIOSIS

Progression of WMH was detected in 28,7% of patients; the most important factor associated was bad clinical control of blood pressure.

In the Austrian Stroke Prevention Study (ASPS) (29) with 273 patients and 3-year of follow-up, with 17,9% of lesion progression, in 9,9% lesion progression was minor and in 8,1% progression was marked; minor was defined by transition to one to four punctate lesions and marked a change to more than four lesions or a transition to early-confluent or confluent. The progression was lower in this study than in our study; one reason could be the differences between samples because the mean age and prevalence of hypertension were lower.

Also factors related to hypertension such as diastolic hypertension showed significant association in ASPS (29).

Progression of WMH was analysed in multinational Leukoaraiosis and Disability study (LADIS) (32) with 396 patients and 3 years of follow up. In this study using the Rotterdam Progression scale (absence/presence of progression in 9 brain regions), the main results were a progression of WMH in 74% of patients and more specifically, 21% of the subjects showed WMH progression in one region and 53% in at least 2 regions. Factors associated with WMH progression were baseline WMH and lacunes, moreover history of diabetes and stroke predicted progression.

In the study of Sabayan et al (525), published in 2015, 534 older patients were examined and WMH was measured 2 times in 33 months automatically, using the Software for Neuro-Image Processing in Experimental Research and the automatically generated WMH segmentations were edited manually later. The median total progression was 0,2 ml/year, in periventricular zone 0,2 ml/year and in deep cortical zone 0,05 ml/year. Rapid progression was associated with age and was linked with subsequent risk of mortality.

5.3 NEW LACUNAR INFARCTS

In our study we detected new IL in 14,9% of the sample during the follow-up. The incidence of lacunes in others studies varied notably, between 0,4-9,5% per year with higher results in hospital-based cohorts. Incidence of lacunes in the hospital-based LADIS and SCANS studies was 5,8% and 9,5% per year respectively (398).

In the Austrian Stroke Prevention Study (29) only 2,2% of new lacunes occurred in 3-years of follow-up in 273 participants with mean age of 60 years.

In the Rotterdam Scan study (30) , that it is a prospective population based study of 1077 subjects 60-90 years, with a follow-up mean of 3,4 years, reported 14% of new silent infarcts (81 silent infarcts and 12 symptomatic infracts). It is important to note that we only consider silent lacunar infarcts in this study.

We obtained similar incidence per year (around 7,5%) than the observed in studies previously referred (LADIS and SCANS) with similar population, and the percentage of Rotterdam study is very similar to our study, taking into account that in the RSS a small percentage of silent stroke were silent cardioembolic and atherothrombotic strokes. One possibility to considerer is that we obtained a lower incidence than SCANS study because our population, despite being at high vascular risk, did not present previously symptomatic SVD , as is the case of the SCANS study where patients had symptomatic LA and confluent LA at basal (528). We probably obtained higher incidence of lacunes than ASPS because our sample is older and with higher percentage of hypertension.

Predictors of progression of IL were mainly the appearance of new MH (OR 20,1; 95% CI 3-135,1); there are important evidences in scientific literature about the interrelation of the progression of the different markers among themselves.

This association was reported previously in Rotterdam Scan Study (442) where pre-existing MH were related to a higher appearance of new LI at follow-up (OR 4,67; 95 % CI 1,84-11,85).

Additionally other phenotypes have been related with the progression of another neuro-imagen biomarker, for example, the severity of basal LA was a predictor in the development of new LI in the LADIS study (398). And in Rotterdam Scan Study a the incidence of new silent infarcts is associated with the presence of previous silent infarcts (OR 2,9; 95% CI 1,7-5.0) (529).

Assessment of lacunes in the majority of studies was realized by manually rated on FLAIR/T2/T1, similar technique than the one used by us (530).

In our study patients with new LI in the follow-up showed significantly higher levels of sTWEAK (OR 1,11; 95% CI 1,06-5,17). sTWEAK is a potential mediator of neuroinflammation, stimulates astrocytes, microglial cells and vascular endothelial cells and induces neuronal cell death. sTWEAK is implicated also in increasing blood-brain barrier permeability and neurovascular unit disruption, most likely via astrocyte expression of MMP-9. Their expression is

upregulated in endothelial dysfunction and disruption of BBB (319). This data could justify our findings.

5.4 PROGRESSION OF MICROHEMORRHAGES

In our study, at basal MRI we detected 10,89% of MH and the prevalence increase to 19,8% at follow-up. We found new MH in 20 patients, 14,9% of the sample.

There are few studies of progression of MH. Rotterdam Scan Study (531), with 831 persons without dementia mean age 68,5 years with an interval of 3,4 years, obtained a 10,2% of new microbleeds. The incidence in Rotterdam Scan Study increased with age from 7,6% in persons aged 60 to 69 years to 18,6% in participants > 80 years. In Rotterdam scan study high systolic blood pressure and hypertension were also associated with an increase in total number of MH. History of hypertension was present in 70,8% of the sample and it was considered severe in only 20% of the patients in Rotterdam Scan Study, while in our sample we have a 99% history of hypertension with a 57,4 % of uncontrolled hypertension; this difference between studies could be may explain the lower results obtained.

Results of new MH during the follow-up of patients with amyloid angiopathy or previous history of stroke reported a higher incidence. In the study leading by Lee with 224 patients with previous history of TIA or stroke over 3 years, an incidence of 0,8 new MH/year was reported and there is another study in patients with possible or probable CAA that reported new MH at 1 year in 46% of participants (532,533).

Patients with progression of MH in the follow-up showed significantly higher serum levels of MMP 7 (OR 1,85; 95% CI 1,03-2,32) and MMP-9 (OR 1,21; 95% CI 1,00-3,12). Previously, metalloproteases (specially MMP-2 and MMP-9) have been implicated in pathogenesis of SVD substrate of subcortical ischemic vascular disease and also in the vascular damage in AD (534).

6. PREDICTORS OF PROGRESSION OF SVD

6.1 VASCULAR RISK PROFILE

We have analysed the degree of control of vascular risk factors with the progression of SVD. To date there are very few works that specifically analyse this aspect.

The vascular risk profile of progression of SVD is mainly uncontrol of hypertension, also in the case of LA specifically. In the rest of secondary variables: progression of cognitive impairment, new LI and new MH we have not obtained statistically significant association.

We have not found a relationship between poor control of diabetes or dyslipidemia and the progression of SVD; in this sense this work is consistent with other previously published studies where they have not found a clear association of the presence of these factors with the progression and additionally in the few studies that analysed association between control of glycemia they didn't find association (494-499).

Surprisingly, obesity is associated with less progression of the disease, but not the waist circumference, which was associated with progression of SVD (535,536). Waist circumference is considered a better biomarker of visceral fat than BMI (537). Visceral fat is truly implicated in poor prognosis or cardiovascular disease Obesity-paradox was amply studied and several factors could explain these potential reasons especially in elderly people such as non-purposeful weight loss, less cachexia, decreasing muscle mass and other confounding factors like not having into consideration the distribution of the fat (507).

We obtained better control of blood pressure, HbA1c levels and LDL in our sample than in registry of prevalence of uncontrolled risk factors in general population. One reason could be the fact that patients visited their primary care physician every 6 months to check the degree of control of their risk factors.

6.2 NEUROIMAGING BIOMARKERS

We have not analysed whether the presence of a certain phenotype, for example basal leukoaraiosis is a marker of progression of any phenotype of the disease, since it is widely documented that the presence of basal phenotypes are markers of risk of progression of SVD (5,528,538)

We have considered investigating if the progress of a certain one can influence the development of another of the phenotypes; in this case the neuroimaging marker that can be considered as a marker of progression of new LI is the appearance of new MH. This is an important finding in relation to secondary prevention of SVD. Usually patients with LI receive antiaggregant treatment however dual antiplatelet therapy did not confer clear benefit (539), specially in patients with SVD because there is a higher risk of ICH (540).

6.3 ULTRASONOGRAPHY BIOMARKERS

Pulsatility and resistance index of the middle cerebral artery are associated with SVD and cognitive impairment (541,542). Ultrasonographic markers of high pulsatility and resistance in our study were not independently associated after being adjusted for other variables. However were higher in the all phenotypes of SVD progression. One reason could be that PI and RI are markers of hypertension damage in the cerebral microcirculation (393).

6.4 BIOCHEMICAL BIOMARKERS

We found correlation between higher levels of circulating fibrinogen and ESR and progression of any SVD phenotype, progression of LA and new LI, supporting the hypothesis that endothelial failure contributes to the pathogenesis of SVD. We didn't find association with progression of MH and cognitive impairment probably due to the small size of the sample.

However, multivariate analyses adjusted for other variables have lost significance, translating that they can be the result of endothelial damage caused by poorly controlled hypertension.

According to previous studies we have found that higher levels of Aβ 1-40 are associated with the progression of SVD and with the progression of cognitive impairment particularly.

In the SVD there is an alteration in the perivascular drainage of the Aβ, diminishing its clearance towards the cephalo- cerebrospinal fluid that later flows into the lymphatic circulation. In addition, Aβ produces breakdown of the BBB, promoting and perpetuating microvascular damage and the accumulation of Aβ and neurotoxicity. Numerous studies suggest Aβ is toxic to the NVU and causes endothelial dysfunction (266–271). Aβ is likely to disrupt the organization of TJs and AJs in endothelial cells, activating MMPs (119,272).

On the other hand, in cases of AD there is an increase in the production of Aβ in addition to a decrease in clearance in cases with microvascular damage as in CAA (521) It is not surprising, therefore, that one of the progression markers of any microangiopathy phenotype and in cases of cognitive deterioration progression is the increase in plasma Aβ levels.

The results in relation to sTWEAK are very important. The increase in levels is a marker of progression mainly of LI , and it is not surprising, so sTWEAK could promote the development of lesions of lipohialinosis and fibrinoid necrosis since it is very related to phenomena of hyperplasia and cell proliferation and rupture of the BBB (321) (543).

From the point of view of MH it is important to consider two factors, that MH are produced by microaneurysms and fibrinoid necrosis suggesting a mechanism of predominant vascular rupture and a percentage of them are due to amyloid angiopathy. In this case we have found that a biomarker of progression is the plasma elevation of metalloproteases MMP-9 and mainly MMP-7. MMP-9 has been related in many research articles to the rupture of the BBB in patients with SVD and with the presence of WMH (544). The relationship of MMP-7 with the progression of MH and SVD has not been described

previously but MMP-7 had been described as very related with the cellular metabolism of Aβ (545).

7. LIMITATIONS OF THE STUDY

We highlight as limitations of the study the following aspects:

1. The sample of the size with a low number of patients included. Many patients refuse to participate. Patients refused to participate because of the distance between their home and the hospital. There was 45km of average.

2. 15 patients abandoned the study, mainly due to transportation difficulties.

2. Short follow-up period compared to other SVD progression studies. Most of these studies has 3 year or more of follow-up (29,546,547)

3. The utilization of 1,5 Tesla MRI. A better quality of MRI could have detected other phenotypes as cerebral cortical microinfarcts (548).

4. In our study we used a visual scale to measure progression grading by Fazekas scale because a previous study reported that visual rating with the Fazekas shows significant correlation with quantitative volumetric assessments and it is also faster to perform (549). However this has not been reported in other studies that recommend using other scales less categorical that can better differentiate the progression between the different grades (550).

In other studies they have used other scales and systems to assess the progression. Visual, automatic or semiautomatic systems have been used (530).

5. Plasma determination of biomarkers of endothelial dysfunction and MMPs may be influenced by subclinical systemic arteriosclerosis; determinations in CSF could have been more specific (544,551)

CONCLUSIONS

1. Asymptomatic neurovascular patients between 60-75 years with hypertension or type 2 diabetes with more than 5 years of evolution have radiological or clinical markers of SVD in 87,1% of cases. The most frequent finding is leukoaraiosis grade I.

2. Our sample shows poorly controlled hypertension in 54%, poorly controlled type 2 diabetes in 46% and poorly controlled dyslipidemia in 35% during the follow-up.

3. Progression of any SVD was detected in 42,6% of patients during the follow-up.

4. Progression of WMH was detected in 28,7% of patients; new lacunar infarcts were detected in 14,9% and new microhaemorrhages were detected in 14,9% also.

5. Poor control of hypertension is associated with a greater progression of WMH.

6. High levels of sTWEAK are associated with a greater development of new LI ; and new MH in patients with previous LI.

7. High levels of MMP-7 and MMP-9 are associated with greater development of new MH.

8. Cognitive impairment was found in 21,8% at basal evaluation but only 2,9% of the sample had cognitive impairment of subcortical profile.

9. 59% of patients developed cognitive impairment. Elevated levels of AB 1-40 are associated with a greater risk of developing cognitive progression in patients with SVD.

10. Bad clinical control of hypertension was the only variable associated with the progression of any SVD phenotype, specially with progression of LA.

10.Uncontrolled diabetes or dyslipidemia were not associated with the progression of SVD.

11.Higher BMI was an independent protective factor for progression of SVD.

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